THE ROLE OF TNF-α IN THE PATHOGENESIS OF *Mycobacterium tuberculosis*

ABSTRACT

THESIS SUBMITTED FOR THE DEGREE OF

Doctor of Philosophy

IN

BIOCHEMISTRY

BY

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THESIS

T-6969

DEPARTMENT OF BIOCHEMISTRY
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The susceptibility of humankind to the deadly parasite *Mycobacterium tuberculosis* has been a grave source of concern throughout history. *M. tuberculosis*, the scourge of humanity, is one of the most successful and scientifically challenging pathogens of all time. The success of this pathogen manifests as tuberculosis (TB), and the extent of its devastation is measured in claiming between 2 and 3 million lives yearly. Tuberculosis is the second leading cause of death worldwide from a single infectious agent. Declared a global health emergency more than a decade ago, tuberculosis resurgence is assuming threatening proportions. Among the important reasons for the causative killer parasite spiralling out of control at an alarming rate is attributed to the emergence of multidrug-resistant strains and the AIDS epidemic.

The initial interaction of mononuclear phagocytes with *M. tuberculosis* gives rise to a cytokine profile dominated by TNF-α. It is well established that both phagocytic and non-phagocytic interaction of *M. tuberculosis* bacilli with mononuclear phagocytes as well as mycobacterial protein and non-protein components can induce this pro-inflammatory cytokine. In the early stages of infection, TNF-α secreted by macrophages is one of the pivotal pro-inflammatory cytokines responsible for disease localization and granuloma formation. Paradoxically, in the more advanced and chronic stage of mycobacterial diseases, TNF-α has been shown to be responsible for disease severity and pathogenesis. Antigen 85B is one of the many *M. tuberculosis* products that induce production of TNF-α. Along with the other two proteins in the *M. tuberculosis* 85 complex (85A and 85C), 85B is abundantly secreted by *M. tuberculosis*.

The resurgence of tuberculosis as a major disease with an estimated 10.2 million new cases in 2005 is fuelling an urgent need for developing novel therapeutic strategies against the causative organism, *M. tuberculosis*. Focus has now shifted to development of compounds from natural sources that have antimycobacterial activity. By boosting host immunologic responsiveness, these compounds may be particularly useful in the treatment of drug-resistant tuberculosis. We studied the incorporation of such compounds, like allicin from garlic, as the herbal component for tuberculosis management.

The initial part of the present study undertook a detailed characterization of sera and monocytes of patients with active tuberculosis to evaluate the expression of TNF-α and *M. tuberculosis* 85B. Direct binding ELISA on microtitre plates coated with protein antigens of H37Rv bacilli sonicate showed remarkable reactivity against antibodies found in sera of tuberculosis patients, as evidenced by an antibody titre >1:12800. Similarly, an appreciable reactivity was observed with tuberculosis sera against secreted culture filtrate protein antigens with an antibody titre >1:6400, whereas no reactivity was observed with normal human sera in either case.

M. tuberculosis infection gives rise to a cytokine profile that is dominated by TNF-α. In our study also, estimation by ELISA showed an appreciable magnitude of soluble TNF-α in sera of tuberculosis patients. Furthermore, *M. tuberculosis* 85B is a predominant protein produced during human *M. tuberculosis* infection; however its role in tuberculosis pathogenesis is not clear and warrants further investigation. The presence of circulating *M. tuberculosis* 85B antigen in sera of tuberculosis patients, either in the free form or complexed with fibronectin has been well established. We also found an appreciably elevated level of antigen 85B in sera of tuberculosis patients.

After characterizing sera, an attempt was made to characterize monocytes from tuberculosis patients with respect to TNF-α and 85B. Monocytes from tuberculosis patients were subjected to TNF-α and 85B mRNA evaluation by real-time RT-PCR and revealed appreciably high basal levels of both mRNA expression. TNF-α and 85B proteins were also found to be significantly elevated in supernatants of monocyte cultures of tuberculosis patients.

Despite encountering a robust immune response, *M. tuberculosis* successfully survives and persists in its human host. Of the various *M. tuberculosis* genes shown to be up regulated after *M. tuberculosis* infection of human mononuclear cells, the 85B gene was expressed most frequently. Antigen 85B is a mycolyl transferase involved in cell wall biosynthesis shown to catalyse the formation of trehalose dimycolate which enhances the host inflammatory response. Thus, in the present study, we investigated the early regulation of *M. tuberculosis* 85B gene by allicin in *M. tuberculosis* cultures.

Real-time RT-PCR data showed that allicin downregulated the expression of 85B mRNA in *M. tuberculosis* (H37Rv) cultures in a dose-dependent manner, without any significant effect on the expression of the housekeeping gene of *M. tuberculosis*, i.e., 16S rRNA. An appreciable suppression of 85B mRNA by ~2 and 2.5 logs was recorded with 250 and 500 ng/ml of allicin, respectively. It is to be pointed out here that previous reports have shown *M. tuberculosis* growth to be affected by allicin at relatively much higher concentrations. From our data it is evident that although low concentrations of allicin that have been employed in this study do not affect *M. tuberculosis* growth, it appreciably downregulates the *M. tuberculosis* 85B gene expression.

The effect of allicin on expression of *M. tuberculosis* antigen 85 complex (30/31 kDa) in *M. tuberculosis* sonic extract as revealed by gradient SDS-PAGE was also found to be dose-dependent. 250 and 500 ng/ml of allicin proved to be a potent inhibitor of antigen 85 complex protein expression. In supernatants obtained from 14 days *M. tuberculosis* cultures receiving various doses of allicin, the secretion of antigen 85 complex was found to be dose-dependent. Allicin decreased the specificity of TB-IgG for *M. tuberculosis* protein antigens as revealed by immunoassays for sonic extract and culture filtrate protein antigens of *M. tuberculosis* co-cultured with allicin against TB-IgG.

Interestingly, antigen 85B is immunodominant and potently induces TNF-α when complexed to fibronectin in mononuclear phagocytes. Therefore, the role of *M. tuberculosis* 85B in intracellular infection may be the maintenance of an inflammatory response. Through sustaining TNF-α activity, the abundant release of *M. tuberculosis* 85B in situ may contribute to the pathogenesis of *M. tuberculosis* infection. The expression of 85B in *M. tuberculosis*-infected monocytes correlates positively with both the amount of secreted TNF-α and subsequent intracellular mycobacterial growth.

It was observed in our study that *M. tuberculosis* infection had no effect on host housekeeping genes such as the R18 gene (18S rRNA) or β-actin gene as revealed by quantitative real-time RT-PCR and RT-PCR data. Also, the kinetics (0–120 hours) of expression of TNF-α and *M. tuberculosis* 85B mRNA in *M. tuberculosis*-infected

monocytes by real-time RT-PCR, RT-PCR and ELISA was investigated. The data revealed that the expression of TNF-α mRNA in infected monocytes as well as secreted TNF-α in culture supernatants was time-dependent, maximal at 24 hours of infection, followed by a substantial decrease at the remaining time intervals of infection.

Furthermore, 16S rRNA expression in *M. tuberculosis*-infected monocytes was observed to linearly increase with time. The expression of *M. tuberculosis* 85B mRNA was found to continue to increase up to 120 hours of infection. The ratio of 85B:16S was also increased with increasing time periods of infection and correlated with 85B mRNA levels at various time periods. Also, in the supernatants of infected monocyte cultures the expression of secreted antigen 85 complex increased with time. Furthermore, the *M. tuberculosis*-infected monocytes exhibited a linear time-dependent decrease in GPx activity in comparison to control monocytes.

The above data suggested a role for endogenous TNF-α in auto-induction and in the induction of *M. tuberculosis* 85B gene expression. To further assess the role of TNF-α, in other experiments, exogenous rhTNF-α and soluble TNF-α receptors were added to *M. tuberculosis*-infected monocytes, and then TNF-α and 85B mRNA were assessed. The cell activation by exogenous rhTNF-α induces both TNF-α and *M. tuberculosis* 85B genes in *M. tuberculosis*-infected monocytes, whereas inhibition of endogenous TNF-α by TNFR-I and TNFR-II was associated with down-modulation of both TNF-α and *M. tuberculosis* 85B gene expression. The results indicate sTNFR-I to be a stronger inhibitor of TNF-α and *M. tuberculosis* 85B gene expression in *M. tuberculosis*-infected monocytes than sTNFR-II.

Cellular activation, and thereby induction of TNF-α, is mediated via NF-κB. It has been well documented that TNF-α induced nuclear translocation of NF-κB was inhibited in SN50 peptide-treated human monocytic cell lines as demonstrated in EMSA. Thus, we employed SN50, an inhibitor of NF-κB, to assess the role of NF-κB in activation of gene expression in *M. tuberculosis*-infected monocytes. Our results clearly show that the increased expression of TNF-α and 85B mRNA in *M. tuberculosis*-infected monocytes is mediated mainly via NF-κB. Interestingly, inhibition of reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates

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(RNIs), reduced expression of TNF-α and *M. tuberculosis* 85B genes in *M. tuberculosis*-infected monocytes.

Allicin (diallyl thiosulfinate) is the major biologically active component and thiosulfinate compound of freshly crushed garlic. It has been reported to enhance the actions of antibiotics such as chloramphenicol and streptomycin against *M. tuberculosis*, besides affecting different biological activities such as antimicrobial, antiparasitic and antifungal activities. Allicin has radical scavenging properties in activated granulocytes and may also inhibit inducible nitric oxide synthase expression in activated macrophages.

Here we investigated the effect of allicin on expression of TNF-α and *M. tuberculosis* 85B in *M. tuberculosis*-infected monocytes after 24 hours of infection. Since higher doses of allicin have previously proven to be toxic by various investigators, the present study employed lower concentrations (0–500 ng/ml), which failed to show any toxic effect on monocytes as revealed by MTT assay. Also, no effect was observed on housekeeping genes: R18 (18S rRNA) by quantitative real-time RT-PCR, or β-actin gene as revealed by RT-PCR, thereby indicating that allicin did not non-specifically affect human TNF-α transcription in *M. tuberculosis*-infected monocytes. Also, the effect of various doses of allicin employed in the present study failed to show any toxic or inhibitory effect on the mycobacterial housekeeping gene *M. tuberculosis* 16S rRNA by quantitative real-time RT-PCR as well as by RT-PCR in monocytes infected with *M. tuberculosis*.

The present study revealed allicin to downregulate the expression of TNF-α gene in *M. tuberculosis*-infected monocytes as well as the secretion of soluble TNF-α in culture supernatants in a dose-dependent manner. The results indicate an appreciable suppression in endogenous TNF-α mRNA expression by ~7.1 logs and 8.1 logs with 250 and 500 ng/ml of allicin, respectively in *M. tuberculosis*-infected monocytes. Furthermore, allicin was also found to suppress the expression of 85B gene and secreted antigen 85 complex in a dose-dependent manner where 250 and 500 ng/ml of allicin were found to be potent inhibitory concentrations in *M. tuberculosis*-infected monocyte cultures after 24 hours of infection. Interestingly, in comparison to the inhibitory effects of allicin with those of NAC, SN50 and anti-TNF-α antibody,

allicin was found to be the most potent inhibitor of TNF-α and *M. tuberculosis* 85B expression in *M. tuberculosis*-infected monocytes.

Our results indicate that the increased expression of TNF-α and 85B mRNA in *M. tuberculosis*-infected monocytes is mediated via activation of NF-κB, as evidenced by the suppression of TNF-α mRNA in the presence of SN50, an inhibitor of NF-κB. SN50/M, an inactive analogue of SN50 failed to show any such effect. In view of it, our data demonstrated that this effect involved inhibition of the NF-κB pathway induced by allicin probably by inhibiting the degradation of IκBα. Since a number of genes involved in inflammatory responses are regulated by NF-κB pathway, thus a high magnitude downregulation of the NF-κB pathway by allicin would predictably reduce the elaboration of NF-κB-mediated TNF-α mRNA expression and, in turn, 85B gene expression.

Glutathione directly reacts with ROS, and glutathione peroxidase (GPx) catalyzes the removal of hydrogen peroxide. Decrease in GPx activity indicates impairment of hydrogen peroxide-neutralizing mechanisms. Here, we observed a decline in GPx activity in *M. tuberculosis*-infected monocytes. Enhancement of GPx activity in *M. tuberculosis*-infected monocyte cultures after addition of NAC, a precursor of the *in vivo* antioxidant glutathione, indicates reversal of impaired neutralizing mechanisms. Surprisingly, here a slightly greater augmentation in GPx activity was observed when allicin was co-cultured instead of NAC, indicating allicin to be an effective natural antioxidant combating ROS, generated as a consequence of cellular activation in *M. tuberculosis*-infected monocytes.

In conclusion, the presence of elevated levels of TNF-α and *M. tuberculosis* 85B in tuberculosis patients is the indication of severity of the disease. The expression of *M. tuberculosis* 85B was downregulated by allicin in a dose-dependent manner in H37Rv cultures. Furthermore, *M. tuberculosis* infection of monocytes leads to a concomitant activation of TNF-α and expression of *M. tuberculosis* 85B gene. *M. tuberculosis* infection of human monocytes initiates a cascade of events whereby cellular activation by TNF-α, RNI, and ROI enhances the expression of *M. tuberculosis* 85B in monocytes. The augmented expression of TNF-α and *M. tuberculosis* 85B gene in *M. tuberculosis*-infected monocytes by cellular activation

and ROS was suppressed by allicin in a dose-dependent manner. The effect of allicin was mediated by suppression of NF-κB. Also, allicin enhanced the GPx activity, which correlated inversely with the downregulation of TNF-α and *M. tuberculosis* 85B in *M. tuberculosis*-infected monocytes. Allicin may prove to be valuable in the containment of *M tuberculosis* and, therefore, be useful as an adjunct in treatment of tuberculosis. These observations strengthen the idea that allicin should be tested in *in vivo* models to evaluate its therapeutic potential in the pathogenesis of tuberculosis.

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Date .....

Approved.....

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Dr. Najmul Islam (Supervisor)

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2006

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Certificate

This is to certify that **Mr. Nazarul Hasan** has carried out this work for Ph.D. Thesis on the topic entitled "The Role of TNF-α in the Pathogenesis of *Mycobacterium tuberculosis*" under my supervision. To the best of my knowledge, this is Mr. Hasan's original work which is suitable for the award of **Ph.D.** degree in **Biochemistry** of the Aligarh Muslim University, Aligarh, India.

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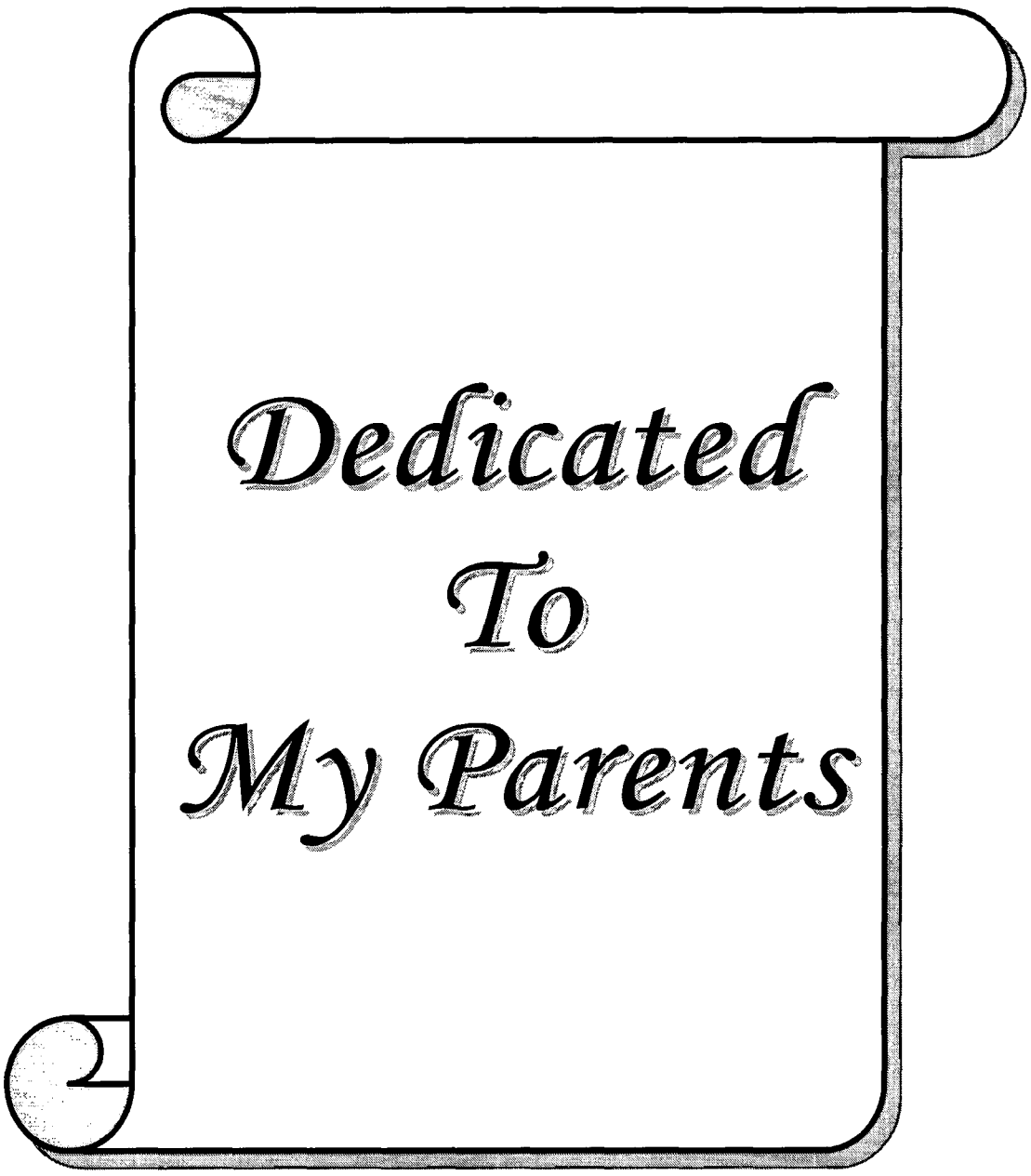

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ACKNOWLEDGEMENTS

In the Name of Allah, Most Gracious, Most Merciful

The Guidance of the Supreme has been the Inspiration behind my Endeavours in this Thesis, from Inception to Completion.

Verily, when He intends A thing,
His Command is "Be",
And it Is!

(Surah Ya-Sin: 82, Qur'an)

I would like to give thanks to the many people whose support has been integral to the accomplishment of this effort.

To my supervisor, ***Dr. Najmul Islam****, for his expert guidance, and for nurturing in me the ability for work, observation and study with his scientific acumen; I am indebted to him for being my mentor and friend, and the expression of it in his cooperation extended at all times. No words can suffice for the depth of gratitude I feel whenever I think of the infinite efforts of his that have gone into the construction of the very fabric of this Thesis; I remain humbled by his magnanimity, witnessed even in the face of his own difficult times, when, undeterred, he would still patiently address each and every concern of mine with an understanding that truly went beyond professionalism, into the realm of generosity of mind and spirit. I thank him from the bottom of my heart for his indispensable role in structuring all my efforts.*

To ***Dr. Mashiat Ullah Siddiqui****, Chairman, Department of Biochemistry, for his moral encouragement and invaluable advice; I will always cherish his endearing ways and words of affection, his charisma and kindheartedness. His gravity of wisdom, and infectious buoyancy of spirit has always given me a profound strength he may be blissfully unaware of, but which has been of inherent value to me at every step of the way, and in crossing every hurdle in my path. I thank him for shielding me in some of my most trying moments.*

To Prof. Rashid Ali, Ex-Chairman, whom it is my duty to acknowledge very humbly for his kind permission to allow me to work in the laboratory at odd hours, often obtained at the cost of his great discomfort and inconvenience.

To all my teachers, Prof. Z.H. Beg, Dr. Khushtar A. Salman, Dr. A.F. Faizy, Dr. Shagufta Moin, Dr. Khursheed Alam and Dr. Moinuddin, for their goodwill, support and help.
I would also like to offer my sincere thanks to Dr. Mohammad Owais, Reader, Interdisciplinary Biotechnology Unit, AMU, for his noteworthy cooperation and constructive counsel whenever I approached him with any query regarding my work, be it large or little.

To all my departmental colleagues, Saba Apa, Jawed, Imran, Manzoor, Khushnood, Zeeshan, Hamida, Nazia, Wahid, Salman, Amir, Prashant, Nadeem and Asad, for their cooperation and teamwork which played an indispensable role in the completion of my work. I also extend sincere

thanks to Dr. Umesh and Dr. Farhan; and especially to Sohail, Dr. Rajeev and Dr. Gaurav for their heart-warming consideration, cooperation and willingness to lend me a helping hand whenever I needed it; for their wonderful company, which became a refreshing oasis for me; and for patiently putting up with all the adjustments to our customary meal-times, and otherwise.

To my friends, Sahil, Shabi, Moonis, Jawed, Wajid, Zafar, and Wahid, for their timely help in my experiments and the related discussions, and for their words and actions of encouragement. In this context, a special mention is due to Farrukh, Atif, Abid, and my brother Sahir, for their help pertaining to different aspects of my experimental work, for their constructive criticism and advice, and for rallying my spirits, all of which has been pivotal to my research during its most crucial phases. My heart goes out to them in deep appreciation of their comradeship.

My fondest expressions of gratitude lie towards my parents and no words I write here will ever do justice to their invaluable prayers for me, their paramount support, constant encouragement, idealism and profound love has been the most important source of comfort and guidance to me. I deeply cherish the wonderful care of my sisters and my brother; their sweet curiosity and appreciation has touched me, reducing the distance between us to insignificance.

At every milestone I cross in my life, my thoughts are never far from the distinct position occupied by my schoolteacher, Mr. Mohammad Fazil; from my formative years to this day I owe a great deal to him for his enduring lessons and his faith in me, which has been seminal in keeping me anchored to the shore at high tide.

I would be failing in my duty, were I not to mention the very kind assistance of all the non-teaching staff of the department, Mr. Behzad, Mr. Shakeel, Mr. Ashfaque and Mr. Faisal, and especially to Mr. Rizwan, Mr. Imran and Mr. Jalil.

Last, but not the least, I wish to express my heartfelt gratitude to aunty (Dr. Zill-e-Huma) and uncle (Dr. Shahid Husain) for their deep concern and guidance at all stages of my work, with utmost respect to Abbu (Prof. Nurul Islam) for his jewels of wisdom and morality which will remain with me life-long, and especially to my uncle (Mr. Riaz Ali Khan) for his fond prayers and expectations, and under the aegis of whom I learnt the ropes of life.

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CONTENTS


Abstract

The susceptibility of humankind to the deadly parasite *Mycobacterium tuberculosis* has been a grave source of concern throughout history. *M. tuberculosis*, the scourge of humanity, is one of the most successful and scientifically challenging pathogens of all time. The success of this pathogen manifests as tuberculosis (TB), and the extent of its devastation is measured in claiming between 2 and 3 million lives yearly. Tuberculosis is the second leading cause of death worldwide from a single infectious agent. Declared a global health emergency more than a decade ago, tuberculosis resurgence is assuming threatening proportions. Among the important reasons for the causative killer parasite spiralling out of control at an alarming rate is attributed to the emergence of multidrug-resistant strains and the AIDS epidemic.

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After characterizing sera, an attempt was made to characterize monocytes from tuberculosis patients with respect to TNF-α and 85B. Monocytes from tuberculosis patients were subjected to TNF-α and 85B mRNA evaluation by real-time RT-PCR and revealed appreciably high basal levels of both mRNA expression. TNF-α and 85B proteins were also found to be significantly elevated in supernatants of monocyte cultures of tuberculosis patients.

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Real-time RT-PCR data showed that allicin downregulated the expression of 85B mRNA in *M. tuberculosis* (H37Rv) cultures in a dose-dependent manner, without any significant effect on the expression of the housekeeping gene of *M. tuberculosis*, i.e., 16S rRNA. An appreciable suppression of 85B mRNA by ~2 and 2.5 logs was recorded with 250 and 500 ng/ml of allicin, respectively. It is to be pointed out here that previous reports have shown *M. tuberculosis* growth to be affected by allicin at relatively much higher concentrations. From our data it is evident that although low concentrations of allicin that have been employed in this study do not affect *M. tuberculosis* growth, it appreciably downregulates the *M. tuberculosis* 85B gene expression.

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The present study revealed allicin to downregulate the expression of TNF-α gene in *M. tuberculosis*-infected monocytes as well as the secretion of soluble TNF-α in culture supernatants in a dose-dependent manner. The results indicate an appreciable suppression in endogenous TNF-α mRNA expression by ~7.1 logs and 8.1 logs with 250 and 500 ng/ml of allicin, respectively in *M. tuberculosis*-infected monocytes. Furthermore, allicin was also found to suppress the expression of 85B gene and secreted antigen 85 complex in a dose-dependent manner where 250 and 500 ng/ml of allicin were found to be potent inhibitory concentrations in *M. tuberculosis*-infected monocyte cultures after 24 hours of infection. Interestingly, in comparison to the inhibitory effects of allicin with those of NAC, SN50 and anti-TNF-α antibody,

allicin was found to be the most potent inhibitor of TNF-α and *M. tuberculosis* 85B expression in *M. tuberculosis*-infected monocytes.

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Glutathione directly reacts with ROS, and glutathione peroxidase (GPx) catalyzes the removal of hydrogen peroxide. Decrease in GPx activity indicates impairment of hydrogen peroxide-neutralizing mechanisms. Here, we observed a decline in GPx activity in *M. tuberculosis*-infected monocytes. Enhancement of GPx activity in *M. tuberculosis*-infected monocyte cultures after addition of NAC, a precursor of the *in vivo* antioxidant glutathione, indicates reversal of impaired neutralizing mechanisms. Surprisingly, here a slightly greater augmentation in GPx activity was observed when allicin was co-cultured instead of NAC, indicating allicin to be an effective natural antioxidant combating ROS, generated as a consequence of cellular activation in *M. tuberculosis*-infected monocytes.

In conclusion, the presence of elevated levels of TNF-α and *M. tuberculosis* 85B in tuberculosis patients is the indication of severity of the disease. The expression of *M. tuberculosis* 85B was downregulated by allicin in a dose-dependent manner in H37Rv cultures. Furthermore, *M. tuberculosis* infection of monocytes leads to a concomitant activation of TNF-α and expression of *M. tuberculosis* 85B gene. *M. tuberculosis* infection of human monocytes initiates a cascade of events whereby cellular activation by TNF-α, RNI, and ROI enhances the expression of *M. tuberculosis* 85B in monocytes. The augmented expression of TNF-α and *M. tuberculosis* 85B gene in *M. tuberculosis*-infected monocytes by cellular activation

and ROS was suppressed by allicin in a dose-dependent manner. The effect of allicin was mediated by suppression of NF-κB. Also, allicin enhanced the GPx activity, which correlated inversely with the downregulation of TNF-α and *M. tuberculosis* 85B in *M. tuberculosis*-infected monocytes. Allicin may prove to be valuable in the containment of *M tuberculosis* and, therefore, be useful as an adjunct in treatment of tuberculosis. These observations strengthen the idea that allicin should be tested in *in vivo* models to evaluate its therapeutic potential in the pathogenesis of tuberculosis.

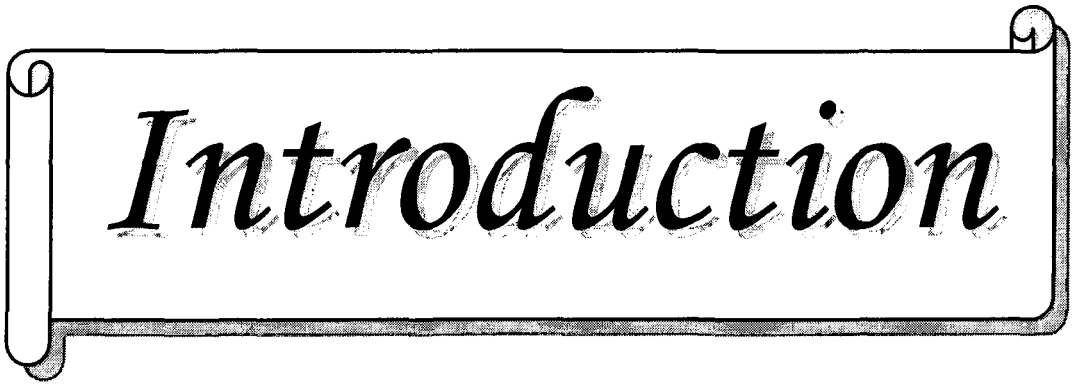
LIST OF ILLUSTRATIONS

LIST OF TABLES

LIST OF ABBREVIATIONS

BCG	:	Bacille Calmette-Guerin
BSA	:	Bovine serum albumin
cDNA	:	Complementary DNA
DEPC	:	Diethyl pyrocarbonate
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxyribonucleotide triphosphate
DOTS	:	Directly Observed Treatment Strategy
DTT	:	Dithiothreiotol
EDTA	:	Ethylene diamine tetra acetic acid
ELISA	:	Enzyme linked imminosorbent assay
EMSA	:	Electrophoretic mobiliy shift assay
GPx	:	Glutathione peroxidase
GSSG	:	Oxidized glutathione
H_2O_2	:	Hydrogen peroxide
IC_{50}	:	Concentration for 50% inhibition
IFN-γ	:	Interferon-gamma
IgG	:	Immunoglobulin G
IL	:	Interleukin
LAM	:	Lipoarabinomannan
LPS	:	Lipopolysaccharide
MDR	:	Multidrug-resistant
MHC	:	Major histocompatibility complex
ml	:	Milliliter
mM	:	Millimolar
MN	:	Monocyte
mRNA	:	Massanger RNA
MTCF	:	*Mycobacterium tuberculosis* culture filtrate
MTSE	:	*Mycobacterium tuberculosis* sonic extract
MTT	:	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NAC	:	N-acetyl cysteine
NADPH	:	Reduced β-nicotinamide adenine dinucleotide phosphate

NF-κB	:	Nuclear factor kappa B
NHS	:	Normal human serum
nM	:	Nanomolar
NMMA	:	N^G-monomethyl-L-arginine-monoacetate
NO	:	Nitric oxide
NOC-9	:	Nonoate-9
oATP	:	Oxidized ATP
PAGE	:	Polyacrylamide gel electrophoresis
PBMC	:	Peripheral blood mononuclear cells
PBS	:	Phosphate buffered saline
PMSF	:	Phenylmethylsulphonyl fluoride
pNPP	:	p-nitrophenyl phosphate
rh	:	Recombinant human
RNA	:	Ribonucleic acid
RNI	:	Reactive nitrogen intermediate
ROI	:	Reactive oxygen intermediate
ROS	:	Reactive oxygen species
RPMI	:	Roswell Park Memorial Institute
rRNA	:	Ribosomal RNA
RT-PCR	:	Reverse transcriptase polymerase chain reaction
SDS	:	Sodium dodecyl sulphate
SN50	:	An inhibitor of NF-κB
SNP	:	Sodium nitroprusside
sTNFR	:	Soluble TNF receptor
TDM	:	Trehalose dimycolate
TEMED	:	N,N,N′,N′-tetramethylethylene diamine
TLR	:	Toll-like receptor
TMM	:	Trehalose monomycolates
TNF-α	:	Tumor necrosis factor-alpha
Tris	:	Tris (hydroxymethyl) aminomethane
µg	:	Microgram
µl	:	Microliter
µM	:	Micromolar


Introduction

Tuberculosis, also called TB, phthisis, consumption, and nicknamed the white plague, is a potentially fatal contagious disease that can affect almost any part of the body, but is mainly an infection of the lungs. It is caused by a bacterial microorganism, the tubercle bacillus or *Mycobacterium tuberculosis*.

Epidemiology:

Tuberculosis is a devastating communicable disease and a global killer as the second leading cause of death worldwide from a single infectious agent (Frieden et al., 2003). Tuberculosis has confounded attempts at control ever since the discovery of *M. tuberculosis* by Robert Koch 120 years ago, for which he was honoured with the Nobel Prize. The staggering extent of infection by this exclusively human pathogen, *M. tuberculosis*, encompasses a total of two billion persons, approximately eight million people develop active disease and nearly two million are killed each year (Barnes and Cave, 2003; Kusner, 2005). Moreover, it was declared a global health emergency by WHO in 1993 (WHO, 2002). Targets were established to measure progress in the implementation of the DOTS (directly observed treatment strategy) to combat tuberculosis by 2005. The targets include 100% population coverage with DOTS, 70% case detection and 85% treatment success rate. In spite of this, the number of new cases of tuberculosis is projected to reach 11.9 million by the end of 2006, if current control efforts are not strengthened (Dolin et al., 1994). Although a cure was available for the second half of the 20th century, various demographic and socioeconomic factors make both prevention and treatment difficult (Tufariello et al., 2003). Most cases are in populous and less developed countries of Asia, with India, China, Indonesia, Bangladesh and Pakistan together accounting for more than half the global burden. The global tuberculosis caseload appears to be growing slowly with increased incidence in the former Soviet Union and Africa (Frieden et al., 2003; WHO, 2003b). The recent resurgence in tuberculosis is attributed partly to the AIDS epidemic with the emergence of multidrug-resistant strains especially in areas where the WHO-recommended DOTS has not yet been implemented (Pablos-Mendez et al., 1998; De Cock and Chaisso, 1999). Co-infection with the human immunodeficiency virus (HIV) and tuberculosis form a lethal combination, each speeding the other's

progress. Sub-Saharan Africa bears the brunt of the HIV-fuelled tuberculosis epidemic (Corbett et al., 2002).

Clinical manifestations:

The most common clinical manifestation of tuberculosis is pulmonary disease. Extrapulmonary tuberculosis accounts for about 20% of disease in HIV-seronegative people, but is more common in HIV-seropositive individuals (Shafer and Edlin, 1996). Among people not infected with HIV, extrapulmonary disease, particularly lymphatic tuberculosis, is particularly common in women and young children (Rieder, 1999). Pleural tuberculosis occurs as a result of either primary progressive *M. tuberculosis* infection or reactivation of latent infection. Unlike other clinical manifestations of tuberculosis, pleural disease probably represents an increased, rather than diminished, immune response (Frieden et al., 2003).

The most serious clinical manifestation of tuberculosis is involvement of the central nervous system. Such involvement can include inflammation of the meninges, as well as space-occupying lesions (tuberculomas) of the brain. The clinical manifestations are due to the presence of *M. tuberculosis* as well as the inflammatory host immune response. Children under 5 years of age and HIV-infected individuals are at increased risk of tuberculous meningitis (Berenguer et al., 1992; Thwaites et al., 2000), which can clinically manifest as chronic meningitis, with headache, fever, and changed mental status. Neurological manifestations can include cranial nerve palsies and motor, sensory, and cerebellar defects, according to the location of the tuberculomas; seizures can also occur. Meningitis is fatal in almost all cases without chemotherapy, and prompt identification and treatment is essential to prevent serious neurological sequelae (Frieden et al., 2003).

Tuberculosis can affect any bone or joint, but the spine (i.e., Pott's disease) is the most common bony structure involved. In the spine, the most common location is the thoracic section. Vertebral-body involvement can be followed by disease of an adjacent intervertebral disc (Iseman, 2000). Genitourinary tuberculosis (including involvement of the renal and male and female genital tracts) is uncommon and is difficult to distinguish from other infections of the genitourinary tract (Frieden et al.,

2003). In men, manifestations include those of prostatitis or prostate enlargement, epididymitis, and orchitis, but disease can also present as a painless scrotal mass. Urine analysis may show red or white blood cells, or both, with a negative urine culture for bacteria (sterile pyuria). In women, genitourinary tuberculosis is an important cause of infertility in areas with high tuberculosis incidence (Goldfarb and Saimn, 1996).

Disseminated tuberculosis is defined as involvement of many organs simultaneously and can occur as a result of primary progressive disease or reactivation of latent infection. The clinical manifestation of pulmonary involvement is a miliary (millet seed) pattern rather than an infiltrate in most cases, but not all patients with disseminated disease have pulmonary involvement (Frieden et al., 2003). Mortality is high despite chemotherapy and may be related to delays in diagnosis and other commonly present underlying medical conditions (Rieder et al., 1990).

Pathophysiology:

The pathogenesis of tuberculosis is complex and its manifestations diverse, reflecting a lifetime of dynamic interactions between mycobacterial virulence factors and the human immune system (Ellner, 1997; Hingley-Wilson et al., 2003). *M. tuberculosis* is an extraordinarily effective human pathogen. Surveys with PPD (purified protein derivative) or positive tuberculin skin tests suggest that one third of the world's population is infected with the bacillus (Dye et al., 1999). Primary infection leads to active disease in only about 10% of infected individuals, mostly within two years (Styblo, 1980). In the remaining 90% majority of cases the immune system contains the infection and the individual is non-infectious and symptom-free. This clinical latency can persist throughout the person's lifetime. Reactivation of latent infection can result when host immune responses are perturbed through advanced immunosuppression (eg., AIDS/HIV infection, use of steroids, malnutrition, etc.) or advanced age (Flynn and Chan, 2001; Tufariello et al., 2003). Reactivation of latent infection contributes substantially to the incidence of adult tuberculosis, especially in more developed countries where disease prevalence is fairly low (Stead, 1967).

Tuberculosis is spread by airborne aerosolized droplet nuclei, which are particles of 1–5 μm in diameter that contain *M. tuberculosis.* Because of their small size, the particles can remain airborne for minutes to hours after expectoration by diseased patients (Wells, 1934; Louden and Roberts, 1966). The infectious droplet nuclei are inhaled and lodge in the alveoli in the distal airways (Frieden et al., 2003). *M. tuberculosis* exhibits specific cellular tropism for mononuclear phagocytes, i.e., alveolar macrophages, monocytes and dendritic cells (Kusner, 2005). *M. tuberculosis* has the remarkable capacity to circumvent destruction within one of the most hostile cell types of a vertebrate host: the macrophage, where they establish a niche to survive and even replicate (Houben et al., 2006). *M. tuberculosis* infection initiates a cascade of events resulting in either successful containment of the infection or progression to active disease (Frieden et al., 2003).

In brief, after being ingested by alveolar macrophages, *M. tuberculosis* replicates and spreads via the lymphatic system to the hilar lymph nodes. The bacterium thus utilizes the host cell as a mechanism of transport into the blood stream and deeper tissues. Cell-mediated immunity usually develops 2–8 weeks after infection. Activated lymphocytes and macrophages form granulomas that limit further replication and spread of the organism (Schluger and Rom, 1998). Although the host response is essential to control of infection, the tubercle bacillus participates in the establishment of latency by using various strategies to evade elimination by the host. The evasion and interaction of pathogenic mycobacteria with the host immune system is discussed in greater detail in the following pages.

An orchestrated series of innate immune pathways, and T-helper 1 (Th1)-dominant adaptive immune pathways are activated following phagocytosis of the bacteria, to culminate in a granuloma at the initial focus of infection (Salgame, 2005). The innate host response is necessary for induction of adaptive immunity to *M. tuberculosis* (Crevel et al., 2002).

(A) Non-specific innate host defense and immune-evasion strategies of *M. tuberculosis*:

The first step in the innate host defense is cellular uptake of *M. tuberculosis,* which involves different cellular receptors and humoral factors. Toll-like receptors

seem to play a crucial role in immune recognition of *M. tuberculosis,* which is the next step. The subsequent inflammatory response is regulated by production of pro- and anti-inflammatory cytokines and chemokines. Effector mechanisms also contribute to innate immunity. Most of the mediators at this point are derived from macrophages and dendritic cells. The initial response determines the local outgrowth, sometimes dissemination of *M. tuberculosis* or containment of infection (Crevel et al., 2002). Phagocytic cells also play a key role in antigen presentation and the initiation of T cell immunity which follows.

(1) Phagocytosis of *M. tuberculosis*:

M. tuberculosis primarily infects macrophages and later dendritic cells and monocyte-derived macrophages by using multiple phagocytic cell surface receptors to gain entry (Aderem and Underhill, 1999). These receptors include the mannose receptors, complement receptors and Fc receptors. Endocytosis involves receptor-binding to non-opsonized *M. tuberculosis* or recognition of opsonins on the surface of *M. tuberculosis* (Crevel et al., 2002). The best characterized receptor for non-opsonin mediated phagocytosis of *M. tuberculosis* is the macrophage mannose receptor, which recognizes terminal mannose residues on mycobacteria (Schlesinger et al., 1996). Phagocytosis through mannose receptors does not trigger O_2^- production (Astarie-Dequeker, 1999) and *M. tuberculosis* exerts an anti-inflammatory signal through this receptor (Nigou et al., 2001). *M. tuberculosis* opsonized with complement factor C3 bind and are taken up through complement receptor 1 (CR1), CR3 and CR4.

Fc receptors, which facilitate phagocytosis of particles coated with IgG antibodies, seem to play little role in tuberculosis (Armstrong and Hart, 1975). However, Fc receptor-mediated phagocytosis is directly linked to an inflammatory response (binding to CR is not) (Aderem and Underhill, 1999). Distinct routes of entry of *M. tuberculosis* may lead to differences in signal transduction, immune activation and intracellular survival of *M. tuberculosis* (Crevel et al., 2002).

(2) Recognition of *M. tuberculosis* – Role of toll-like receptors:

The initial interaction of *M. tuberculosis* with phagocytes through toll-like receptors (TLRs) can affect induction of the adaptive response by inflammatory

cytokine production as well as begin the process of bacterial containment by induction of antimycobacterial functions (Flynn and Ernst, 2000). It is partly through this interaction with TLRs that macrophages produce inflammatory cytokines and chemokines that serve as signals of infection (Tufariello et al., 2003). TLRs mediate the activation of cells of the innate immune system, resulting in destruction of invading microbes through the activation of several signalling cascades. TLRs are phylogenetically conserved mediators of innate immunity, which are essential for microbial recognition on macrophages and dendritic cells (Belvin and Anderson, 1996; Medzhitov et al., 1997). Activation of TLRs is an important link between innate cellular responses and the subsequent activation of adaptive immune defenses against microbial pathogens (Salgame, 2005).

TLR-signalling leads to the nuclear translocation of NF-κB (nuclear factor-κB). NF-κB is a transcription factor involved in the expression of many immune response genes such as those encoding the cytokines TNF-α and interleukins (Houben et al., 2006). TLR-signalling also triggers differentiation of monocytes into macrophages and dendritic cells, thereby generating the cellular populations necessary for a potent innate and adaptive immune response (Krutzik et al., 2005).

Members of the TLR family are transmembrane proteins containing repeated leucine-rich motifs in their extracellular domains, similar to other pattern-recognizing proteins of the innate immune system (Crevel et al., 2002). The cytoplasmic domain of TLR is homologous to the signalling domain of IL-1R (IL-1 receptor) and links to IRAK (IL-1R associated kinase), a serine kinase that activates transcription factors like NF-κB to signal the production of cytokines (Oddo et al., 1998).

Through TLRs *M. tuberculosis* lysate or soluble mycobacterial cell wall-associated lipoproteins induce production of IL-12, a strong pro-inflammatory cytokine (Brightbill et al., 1999). Myeloid differentiation protein 88 (MyD88), a common signalling component that links all TLRs to IRAK (Oddo et al., 1998), was found to be essential for *M. tuberculosis*-induced macrophage activation (Underhill et al., 1999). From several lines of evidence it has become clear that phagocytosis does not lead to immune activation in the absence of functional TLRs (Crevel et al., 2002).

Besides generating cytokine activity, TLR stimulation in macrophages upregulates phagocytosis of bacteria (Doyle et al., 2004) and apoptotic cells (Shiratsuchi et al., 2004). TLRs also appear to regulate fusion events between phagosomes and lysosomes (Doyle et al., 2004). Whether or not pathogenic mycobacteria interfere with TLR signalling to block their delivery to phagosomes, eg., through modulation of MAPK signalling pathways remains to be established *in vivo* (Houben et al., 2006).

(3) Effector mechanisms for killing of *M. tuberculosis*:

(a) Avoidance of macrophage-mediated killing:

Although macrophages provide an effective initial barrier against infection by bacterial pathogens, *M. tuberculosis* has evolved numerous strategies that allow it to survive and set up initial residence within these cells (Zahrt, 2003) after gaining entry via multiple cell surface receptors (eg., mannose receptors, complement receptors and Fc receptors). Pathogenic mycobacteria coat themselves with a serum protein that is recognized by complement, thereby avoiding host inflammatory response (Caron and Hall, 1998). *M. tuberculosis* crosses the first line of defense, i.e., the alveolar barrier by invading the alveolar epithelial cell and secondarily, the endothelial layer, or it does so within infected macrophages (Bermudez and Sangarib, 2001). *M. tuberculosis* evades the innate antimicrobial defenses of macrophages by inhibiting the maturation of its phagosome to a bactericidal phagolysosome (Kusner, 2005). Mycobacterium-induced inhibition of phagosome-lysosome fusion has been considered the central feature of tuberculous pathogenesis, providing the organism with a means to avoid both the direct antimicrobial activity of the innate immune system as well as effective antigen presentation by inhibiting MHC class II dependent antigen presentation pathway and elicitation of adaptive immunity (Vergne et al., 2003; Kusner, 2004). Thus, the survival mechanisms or pathogenic strategies involved include inhibition of phagosome maturation and acidification, modification of the normal phagosomal trafficking pathway, and alteration of recruitment or association of various proteins to the phagosomal membrane. These are discussed below:

Inhibition of phagosome maturation and the consequent intracellular survival of *M. tuberculosis* occurs via inhibition of macrophage Ca^{2+}-mediated signal

transduction through inhibition of macrophage sphingosine kinase (Kusner, 2005). Many lipid components of the mycobacterial cell wall have essential roles in the innate ability of mycobacteria to block the fusion of the bacterial phagosome to lysosomes (Houben et al., 2006). Altered phagosomal maturation is associated with alteration in the protein content of the vacuole including altered Rab GTPase composition (Clemens et al., 2000), exclusion of the vacuolar protein ATPase with consequent lack of acidification (Sturgill-Koszycki et al., 1994), and retention of a protein designated TACO (tryptophan aspartate rich coat protein) (Ferrari et al., 1999). Whereas some mycobacterial cell wall lipids, such as trehalose mycolates, influence the inflammatory response (Geisel et al., 2005; Rao et al., 2005), other components modulate trafficking inside the macrophage. For example, the *M. tuberculosis* PtdIns-3P analog, glycosylated phosphatidyl inisitol lipoarabinomannan (ManLAM), blocks phagosome maturation (Fratti et al., 2003) and inhibits the rise in Ca^{2+} concentration in the macrophage cytosol upon phagocytosis of mycobacteria.

(b) Role of nitric oxide (NO):

Putative and effective host defense mechanisms by innate immune cells to *M. tuberculosis* within the phagolysosomes of activated macrophages include the production of reactive oxygen (ROI) and reactive nitrogen intermediates (RNI). Catalytic action of the respiratory burst by the NADPH-oxidase complex produces ROI such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical ($OH^·$) (Chan et al., 2001). NO is a non-specific, chemically reactive molecule that is important in host defense against a wide variety of microbial pathogens. Although controversial, there is a growing body of evidence that NO produced by *M. tuberculosis*-infected human macrophages and epithelial cells is also antimycobacterial against *M. tuberculosis* (Rockett et al., 1998; Jagannath et al., 1998). Human macrophages produce NO from increased iNOS activity (Chan et al., 2001) as in human lung epithelial cells (Robbins et al., 1994). TNF-α and IL-1β, along with IFN-γ produced by T lymphocytes, can induce NO in macrophages via inducible form of the enzyme nitric oxide synthase (iNOS) (Kwon, 1997). Mycobacterial cell wall components such as LAM and 19 kDa lipoprotein, along with T cell-derived IFN-γ may also induce NO expression (Adams et al., 1993; Brightbill et al., 1999).

(c) Apoptosis:

Apoptosis of infected phagocytic cells may prevent dissemination of infection by reducing viability of intracellular *M. tuberculosis* and limiting its outgrowth (Keane et al., 1997; Placido et al., 1997). NO may induce such apoptosis (Chan et al., 2001). TNF-α is also required for induction of apoptosis in response to *M. tuberculosis* infection (Keane et al., 1997). Another potential mechanism for apoptosis-induced mycobacterial killing is that, as with host nuclear fragmentation, mycobacterial DNA may also be destroyed during apoptosis (Molloy and Kaplan, 1996). The selective induction of IL-10 by *M. tuberculosis* may lead to decreased TNF-α activity and reduced apoptosis of infected cells (Astarie-Dequeker et al., 1999). In addition, increased expression of Fas ligand in infected macrophages may also contribute to decreased macrophage apoptosis (Mustafa et al., 1999).

(4) Cytokine production driven by *M. tuberculosis*:

Cytokines are a group of hormone-like polypeptides that play a variety of regulatory roles in host defense against infection (Poveda et al., 1999). Once infection is established, a focal nonspecific inflammatory response follows. This response is regulated by a network of pro- and anti-inflammatory cytokines and chemokines (Crevel et al., 2002), which serve as signals of infection (Means et al., 1999). There are two kinds of cytokines, pro-inflammatory and anti-inflammatory. The pro-inflammatory cytokine network plays a crucial role in the inflammatory response and the outcome of mycobacterial function. This response is antagonized by anti-inflammatory mechanisms. In addition some cytokines may inhibit the production or the effects of pro-inflammatory cytokines in tuberculosis (Crevel et al., 2002).

(a) Pro-inflammatory cytokines:

(i) *TNF-α:*

Stimulation of monocytes, macrophages and dendritic cells with mycobacteria or mycobacterial products induces TNF-α production (Henderson et al., 1997; Valone et al., 1988). This prototype pro-inflammatory cytokine plays a key role in granuloma formation (Senaldi et al., 1996), induces macrophage activation and has immunoregulatory properties (Tsenova et al, 1999). However, systemic spillover of

TNF-α may account for unwanted inflammatory effects like fever and wasting (Crevel et al., 2002). Thus, TNF-α has both adverse and beneficial effects in the human immune response (Tufariello et al., 2003). To limit the deleterious effects of TNF-α (Hernandez and Rook, 1994; Bekker et al., 2000), systemic production of TNF-α is downregulated (Friedland et al., 1995; Takashima et al., 1990). In spite of this, anti-TNF-α therapy in the treatment of tuberculosis is associated with an increased risk of developing tuberculosis (Keane and Gershon, 2002), mainly due to reactivation of a chronic infection (Keane et al., 2001). In addition, TNF-α has an important role in the maintenance of the granulomatous response in latent tuberculosis, and in maintaining the organization of the granuloma, the integrity of which contributes to the containment of *M. tuberculosis* (Tufariello et al., 2003).

(ii) *IL-1β:*

This pro-inflammatory cytokine is produced mainly by monocytes, macrophages and dendritic cells (Dahl et al., 1996; Gerosa et al., 1999). It is expressed in excess (Schauf et al., 1993) and at the site of disease in tuberculosis patients (Bergeron et al., 1997). IL-1R antagonist (IL-1Ra) is the naturally occurring antagonist of IL-1. It has been suggested that an increased IL-1β/IL-1Ra ratio protects against a more severe presentation of tuberculosis, especially tuberculosis pleuritis (Wilkinson et al., 1999).

(iii) *IL-6:*

This is an early cytokine, which has both pro- and anti-inflammatory properties (VanHeyningen et al., 1997) and is produced at the site of infection (Hoheisel et al., 1998). IL-6 may be harmful in mycobacterial infections, as it inhibits the production of TNF-α and IL-1β (Schindler et al., 1990). However, other reports indicate a protective role for IL-6, which seems related to optimal IFN-γ production early in *M. tuberculosis* infection (Ladel et al., 1997a).

(iv) *IL-12:*

IL-12 is produced mainly by phagocytic cells after phagocytosis of *M. tuberculosis* (Ladel et al., 1997b). It is an important early pro-inflammatory cytokine that drives the production of IFN-γ from natural killer (NK) cells (Trinchieri, 2003). Thus, IL-12 is the inducer cytokine and IFN-γ is the effector cytokine that mediates

protection (Ismail et al., 2002) as discussed further. IL-12 is a regulatory cytokine that connects the innate and adaptive host response to mycobacteria (Trinchieri, 1995) and which exerts its protective effects mainly through the induction of IFN-γ (Cooper et al., 1997). In tuberculosis, IL-12 has been detected in lung infiltrates (Taha et al., 1997), and in granulomas (Bergeron et al., 1997). The expression of IL-12 receptors is also increased at the site of disease (Zhang et al., 1999). Three additional cytokines with IL-12-like activities have been identified, notably IL-18, IL-23 and IL-27 (Vosse et al., 2004). IL-18, a novel pro-inflammatory cytokine shares many features with IL-1 (Dinarello et al., 1998) and acts as an IFN-γ inducing factor, synergistic with IL-12 (O'Neill and Greene, 1998). It also stimulates the production of other pro-inflammatory cytokines, chemokines, and transcription factors (Netea et al., 2000). IL-15 is similar to IL-2 in its biological activities and stimulates T cell and NK cell proliferation and activation (Kennedy and Park, 1996). However, unlike IL-2, the production of IL-15 is mainly by monocytes and macrophages (Crevel et al., 2002).

(v) *IFN-γ:*

An important macrophage-activating molecule involved in the immune defense against pathogenic mycobacteria is interferon-γ. Production of IFN-γ is regulated by other cytokines, particularly IL-12. In the early phase of the immune response, IL-12 drives the production of IFN-γ from NK cells (Trinchieri, 2003). Several immune mechanisms, such as antigen presentation, leukocyte-endothelium cell interactions, cell growth and apoptosis, reactive nitrogen and oxygen intermediates as well as phagosome-lysosome fusion can be modulated through the activity of IFN-γ (Houben et al., 2006). Activation of macrophages by IFN-γ also induces autophagy, which is a normal degradative pathway implicated in innate immune mechanisms against *M. tuberculosis* (Ogawa et al., 2005).

(b) Anti-inflammatory cytokines:

The pro-inflammatory response is antagonized by anti-inflammatory mechanisms. Soluble cytokine receptors prevent binding of cytokines to cellular receptors, blocking further signalling (Crevel et al., 2002). Anti-inflammatory cytokines are also important in immuno-regulation.

THESIS

(i) *IL-10:*

IL-10 is produced by macrophages after *M. tuberculosis* phagocytosis (Shaw et al., 2000) and after binding of LAM (Dahl et al., 1996), by dendritic cells and macrophages (Tufariello et al., 2003). IL-10 downregulates the production of the pro-inflammatory cytokines IFN-γ, TNF-α and IL-12 (Fulton et al., 1998; Hirsch et al., 1999). IL-10 can deactivate macrophages and dampen the immune response to prevent or limit pathology from an over-exuberant inflammatory response to a pathogen. This cytokine may have a role in the chronic phase of infection, since downregulation of a type I immune response is likely to be beneficial to the host, at least in terms of lung pathology (Tufariello et al., 2003). IL-10 expression in tuberculosis has been demonstrated in circulating mononuclear cells, at the site of disease in pleural fluid and in alveolar lavage fluid (Barnes et al., 1993; Gerosa et al., 1999).

(ii) *TGF-β:*

TGF-β is produced by monocytes and dendritic cells following induction by mycobacterial products (Toossi et al., 1995). TGF-β suppresses cell-mediated immunity; in T cells it inhibits proliferation and IFN-γ production; in macrophages it antagonizes antigen presentation, pro-inflammatory cytokine production, and cellular activation. TGF-β may be involved in tissue damage and fibrosis during tuberculosis, as it promotes the production and deposition of macrophage collagenases (Toossi and Ellner, 1998).

(iii) *IL-4:*

IL-4 has deleterious effects in tuberculosis, which have been ascribed to this cytokine's suppression of IFN-γ production (Powrie and Coffman, 1993) and macrophage activation (Appelberg et al., 1992).

(5) NF-κB activation – Role in innate immunity:

It is well established that the classical NF-κB pathway, based on IKKβ-dependent IκB degradation, is essential for innate immunity (Bonizzi and Karin, 2004). The activation and nuclear translocation of classical NF-κB dimers is associated with increased transcription of genes encoding chemokines, cytokines, adhesion molecules [intracellular adhesion molecule-1 (ICAM-1), vascular cell

adhesion molecule-1 (VCAM-1) and endothelial-leukocyte adhesion molecule-1 (ELAM-1)], enzymes that produce secondary inflammatory mediators and inhibitors of apoptosis (Ghosh et al., 1998). These molecules are important components of the innate immune response to invading microorganisms and are required for migration of inflammatory and phagocytic cells to tissues where NF-κB has been activated in response to infection or injury (Bonizzi and Karin, 2004). Indirect pathways that lead to NF-κB activation are illustrated by infection of pulmonary epithelial cells with *M. tuberculosis*, which results in the release of IL-1 and activation of the classical NF-κB pathway in adjacent cells (Wickremasinghe et al., 1999).

(B) Specific acquired/adaptive immune response:

(1) T-cell mediated immunity:

In most infected individuals, cell-mediated immunity develops 2–8 weeks after infection. The development of cell-mediated immunity is associated with the development of a positive result in the tuberculin skin test (Frieden et al., 2003). Macrophages and dendritic cells, the primary cell types involved in the innate immune response to *M. tuberculosis*, play a crucial role in the initiation of adaptive immunity (Crevel et al., 2002). The initial interaction of *M. tuberculosis* with phagocytes through TLRs can affect induction of the adaptive response by inflammatory cytokine production, as well as begin the process of bacterial containment by induction of antimycobacterial functions. The initiation of the adaptive immune response to the organism is likely to be a major determinant of long-term control of the infection (Flynn and Ernst, 2000). Thus, TLRs are an important link between innate and adaptive immunity (Duin et al., 2005). The significance of TLRs in activating adaptive immunity is well established (Iwasaki and Medzhitov, 2004); TLR-mediated activation of dendritic cells is a crucial step in this process. Other mechanisms involved include induction of expression of cytokines and co-stimulatory proteins (Duin et al., 2005). Signalling through TLRs favours the development of a Th1-type response, in which IL-12 has an important role (Trinchieri, 2003).

$CD4^+$ T cells have a crucial role in protection against tuberculosis (Selwyn et al., 1989; Tufariello et al., 2003). In human beings, $CD8^+$ T cells have been reported to

be involved in the control of both acute and chronic infection (Rolph et al., 2001; Turner et al., 2001). $CD8^+$ T cells specific for *M. tuberculosis* can directly kill intracellular bacilli via a granule-associated protein, granulysin (Stenger et al., 1998).

In brief, at the focal site of infection in the lungs, the dendritic cells that engulf bacteria mature (Hertz et al., 2001; Bodnar et al., 2001) and migrate to the required lymph node. Once there, $CD4^+$ and $CD8^+$ T cells are primed against mycobacterial antigens. Primed T cells expand and migrate back to the lungs and through the lung tissue to the focus of infection, presumably in response to signals such as chemokines produced by or in response to infected cells (Tufariello et al., 2003). *M. tuberculosis*-infected macrophages release IL-12 and IL-18 which stimulate the $CD4^+$ and $CD8^+$ T cells to release IFN-γ (Sodhi et al., 1997; Ellner, 1997). IFN-γ is crucial for the control of *M. tuberculosis* infection (Flynn et al., 1993). It stimulates phagocytosis of *M. tuberculosis* and also the release of TNF-α from macrophages, which is important in granuloma formation and control of the extent of infection (Flynn et al., 1995; Bean et al., 1999).

Three processes contribute to the infection of adaptive immunity: antigen presentation, costimulation, and cytokine production. These are discussed below:

(a) Antigen presentation:

The T lymphocyte response is antigen specific and is influenced by the major histocompatibility complex (MHC) (Flynn and Ernst, 2000). MHC class II molecules are expressed on professional antigen-presenting cells (APCs). They present mycobacterial proteins to antigen-specific $CD4^+$ T cells. These antigens are processed in the phagolysosomes of APCs (Crevel et al., 2002). MHC class I molecules are expressed on all nucleated cells. They present mycobacterial proteins to antigen-specific $CD8^+$ T cells. This is important for the presentation of cytosolic antigens, which may be important as certain mycobacteral antigens may somehow escape the phagosome (Mazzaccaro et al., 1996).

Pro-inflammatory cytokines, primarily IFN-γ stimulate MHC expression, whereas anti-inflammatory cytokines inhibit its expression. Polymorphism of MHC may contribute to differences in disease susceptibility or outcome (Goldfeld et al., 1998). The expression of particular class I and class II MHC alleles in an individual

determines mycobacterial antigens and epitopes (Crevel et al., 2002). Certain allelic human leukocyte antigen variants (HLA polymorphism) have been associated with tuberculosis and susceptibility to it (Goldfeld et al., 1998; Ravikumar et al., 1999).

(b) Costimulation:

Maturation of dendritic cells induced by TLR stimulation results in accumulation of antigen-MHC II complexes on the cell surface, thus facilitating antigen presentation (Cella et al., 1997; Hertz et al., 2001). Antigen presentation only leads to T cell stimulation in the presence of particular costimulatory signals. The co-stimulatory proteins CD80 (Kindler et al., 1989), CD86 (Sarno et al., 1991) and CD70 are upregulated on antigen-presenting cells after TLR activation (Iwasaki and Medzhitov, 2004). These molecules (CD80 and CD86) bind to CD28 and CTLA-4 on T cells (Crevel et al., 2002). In the absence of proper co-stimulatory signals, antigen presentation may lead to increased apoptosis of T cells (Hirsch et al., 2001).

(c) Cytokine production:

Cytokine production by activated macrophages and dendritic cells is important for T cell stimulation. These cytokines include IL-12, IL-18, and IL-23 as well as IL-1 and TNF-α (Dinarello, 1996; Tsenova et al., 1999; Oppmann et al., 2000). IL-12 is the inducer cytokine that mediates protection (Ismail et al., 2002). During the ensuing immune response, IL-12 plays a key part in driving the activation, differentiation from $CD4^+$ T cells, and expansion of antigen-specific Th1 cells. These cells are a major source of IFN-γ during the adaptive immune response and are necessary for the control of the chronic phase of infection (Janeway and Medzhitov, 2002). The activation of infected macrophages by IFN-γ in synergy with TNF-α is a major effector mechanism of cell-mediated immunity. *M. tuberculosis* induces TNF-α synthesis by a TLR2-dependent pathway (Underhill et al., 1999). IFN-γ, together with TNF-α, which is released by T cells and phagocytes, activates microbicidal mechanisms in mononuclear phagocytes (Vosse et al., 2004).

(2) Granuloma formation:

The granuloma is a hallmark protective immunopathological response of the host following infection with *M. tuberculosis*. It is considered to be a protective

structure since it curtails the spread of the pathogen and at the same time localizes inflammation and damage to the lungs (Salgame, 2005). Primed T cells in the lymph nodes expand and migrate back to the lungs and then through the lung tissue to the focus of infection in response to chemokine signals especially TNF-α. This culminates in the formation of a granuloma, a characteristic feature of tuberculosis (Tufariello et al., 2003). Besides T cells and infected macrophages, additional immune host cells are recruited, including B cells, dendritic cells, endothelial cells, fibroblasts, and probably stromal cells (Gonzalez-Juarrero et al., 2001). The granuloma encompasses the bacilli, which reside within macrophages, and serves to wall off the bacteria from the rest of the lung, limiting spread. In addition, the granuloma functions as an immune microenvironment to facilitate interactions between T cells, macrophages and cytokines. However, the granuloma can also provide a home for *M. tuberculosis* for an extended period, because some bacteria can avoid elimination within the granuloma (Tufariello et al., 2003). The granuloma subsequently develops central areas of necrosis (called caseum, from the word 'cheese'), resulting in the death of the majority of the bacteria and destruction of the surrounding host tissue. The surviving bacilli exist in a latent state and can become reactivated to develop active disease (Grosset, 2003). Proteosome-mediated inactivation of NO stress is important for the survival of mycobacteria within granuloma structures (Darwin et al., 2003; Pieters and Ploegh, 2003). Although excessive replication of bacteria seems to result in loss of granuloma structure, extensive necrosis, and cavity formation, in most cases the granuloma contains the infection, but a limited number of bacilli survive, which is termed latent tuberculosis. A person who has a delayed type hypersensitivity response to mycobacterial antigens (PPD) without signs of active tuberculosis is deemed to have latent infection and not to be contagious.

The Bacterium:

The causative organism of tuberculosis, *Mycobacterium tuberculosis* is a Gram-positive aerobic bacterium that divides every 16–20 hours. It is a small rod-like bacillus which can withstand weak disinfectants and can survive in a dry state for weeks but can only grow within a host organism.

(A) *M. tuberculosis* cell envelope – Architecture and composition:

M. tuberculosis is surrounded by a complex envelope of unusually low permeability, which contributes to the resistance of this bacterium to host defense mechanisms (Jarlier and Nikaido, 1994; Daffé and Draper, 1998). In general, the bacterial cell envelope is composed of a plasma membrane, a complex wall comprising a variety of covalently and non-covalently linked carbohydrates and lipids, and finally an outer layer (capsule) of polysaccharides, proteins and small amounts of lipids. The plasma membrane is a typical bilayer of proteins and phospholipids (Daffé and Draper, 1998). Similar to classical bacterial cell walls, the cell envelope of *M. tuberculosis* contains a cell membrane and a peptidoglycan layer (Glickman and Jacobs, 2001). The thick peptidoglycan layer is covalently linked to a heteropolysaccharide, namely arabinogalactan. However, in other aspects, the *M. tuberculosis* cell envelope differs substantially from the canonical cell wall structures of both Gram-negative and Gram-positive bacteria (Fig. 1). The cell envelope is typified by the presence of a large hydrophobic layer of unusual long-chain fatty acids: mycolic acids, esterified to the cell wall, named the mycolyl arabinogalactan (mAG) (Glickman and Jacobs, 2001). Mycolic acids are long chain (60–90 carbon atoms), branched, α-β hydroxyl fatty acids that exist either covalently attached to the cell wall or non-covalently attached in the form of trehalose dimycolate (TDM). The arabinan segments of arabinogalactan in the peptidoglycan layer are terminated by a hexa-arabinosyl unit esterified by the various types of mycolic acid residues elaborated by the species (Daffé, 2000). These long-chain fatty acids are probably arranged into an asymmetric bilayer with other non-covalently linked lipids, which forms a virtually impermeable outer barrier obstructing access of hydrophilic substances. Mycolic acids are thought to play an important role in the structure of the cell wall and to contribute to the resistance of mycobacteria to many therapeutic agents (Jarlier and Nikaido, 1994). Among the non-covalently linked lipids that can be found in the outer bilayer are TDM, trehalose monomycolates (TMM), glycerol monomycolates and phospholipids. A wide array of unique lipids and glycolipids are non-covalently associated with the cell envelope and confer extreme hydrophobicity to the outer surface of the organism (Daffé, 2000).

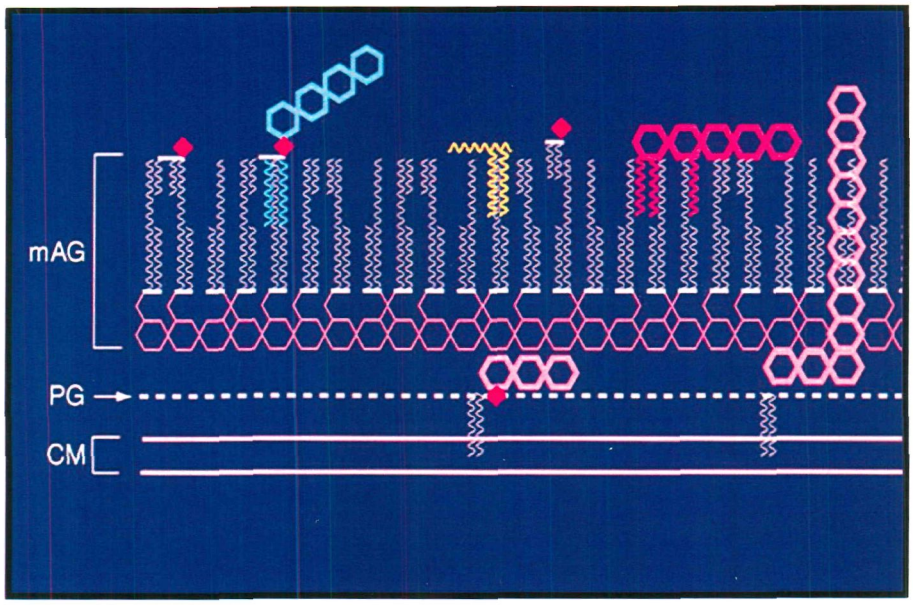


Figure 1. **Schematic Diagram of the Cell Envelope of *M. tuberculosis*: The cell envelope of *M. tuberculosis* contains a cell membrane (CM) and a peptidoglycan layer (PG) similar to those of classical bacterial cell walls. In contrast to other bacteria, *M. tuberculosis* has a thick hydrophobic layer of mycolic acids esterified to the cell wall, named the mycolyl arabinogalactan (mAG). (Source: Glickman and Jacobs. Cell. 2001 104: 477–485.)**

Many of these molecules are potent immunomodulators, suggesting a role in virulence. For example, lipoarabinomannan, a major cell wall-associated glycolipid, has been extensively examined *in vitro* and can inhibit IFN-γ activation of macrophages (Sibley et al., 1988; 1990), induce TNF-α release from macrophages (Chatterjee et al., 1992), and scavenge oxygen free radicals (Chan et al., 1991). TDM, a mycolic acid-containing glycolipid, can produce granulomatous inflammation and thymic atrophy when injected into mice (Ozeki et al., 1997), in addition to affecting membrane fusion in model systems (Spargo et al., 1991).

Like Gram-positive bacteria, the mycobacterial cell envelope contains proteins, including some with pore-forming ability (Daffé, 2000). The increasing number of enzymes recently identified as being involved in cell envelope biogenesis includes the three predominant secreted proteins of *M. tuberculosis*, antigens 85A, 85B and 85C (Wiker and Harboe, 1992).

(B) *M. tuberculosis* antigen 85 complex:

The antigen 85 complex consists of three internally cross-reacting antigens encoded by three independent genes, *fbpA, fbpB, fbpC,* found in the genomes of *M. tuberculosis*, *M. leprae* and *M. avium* (Wiker et al., 1990; Content et al., 1991; Daffé, 2000). In antigen 85 complex, the gene for antigen 85A encodes a 294 amino acid mature protein (Borremans et al., 1989), which shows 79% homology with the deduced 285 amino acid sequence of antigen 85B (Matsuo et al., 1988). The antigen 85 complex is often referred to as the 30/31 kDa doublet and the molecular masses of the individual components of the antigen 85 complex are as follows: antigen 85B, 30 kDa; antigen 85A, 31 kDa; antigen 85C, 31.5 kDa. Antigen 85C appears to be slightly heavier than antigen 85A, and in most SDS-PAGE runs these two components are not properly resolved (Wiker and Harboe, 1992). Thus, the pattern in Western immunoblotting usually reveals a doublet in the 30 kDa region, as exemplified by using the monoclonal antibody HYT27 (Schou et al., 1985) which reacts with all three components of the complex (Wiker et al., 1992). The components are clearly resolved in isoelectric focusing, providing three distinct bands (Vooren et al., 1991). In *M. tuberculosis* H37Rv culture fluid obtained from a batch with minimal cell lysis, the

quantitatively dominating components of the antigen 85 complex, antigens 85A and 85B, constituted about 60% of total protein and there was slightly more antigen 85B than antigen 85A. Although antigen 85 complex is a major constituent of the mycobacterial culture fluid, it is also found in association with the bacterial surface. The monoclonal antibody HYT27 gave a positive signal in ELISA with whole *M. tuberculosis* cells (Schou et al., 1985). The localization index for antigen 85A has consistently been found to be higher than for antigen 85B, indicating that antigen 85B may be less efficiently secreted and more closely associated with the bacterial surface (Wiker et al., 1991; Wiker and Harboe, 1992). Worsaae et al. (Worsaae et al., 1988) performed skin testing with the antigen 85 complex affinity purified with monoclonal antibody HYT27. The available results indicate that antigen 85B is a relatively good skin test reagent whereas antigen 85A is not. The information on antigen 85C remains to be established (Wiker and Harboe, 1992).

Antigen 85B is a mycolyl transferase involved in cell wall biosynthesis. In an *in vitro* enzymatic assay, antigen 85B has been shown to catalyse the transfer of a mycolyl residue from one molecule of TMM to another, leading to the formation of TDM (or cord factor) (Belisle et al., 1997). It stimulates lymphocyte blastogenesis and IFN-γ production in healthy individuals, but not in patients with pulmonary tuberculosis (Huygen et al., 1988; Havlir et al., 1991). In contrast, antibody to the 85B antigen is increased in patients with tuberculosis (Torres et al., 1994). Interestingly, antigen 85B is immunodominant (Abou-Zeid et al., 1988) and potently induces TNF-α when complexed to fibronectin in mononuclear phagocytes (Aung et al., 1996). Fibronectins are a family of high molecular weight glycoproteins found in plasma and tissues that are involved in cell motility and adhesion, regulation of cell morphology, phagocytic function, and wound healing (Proctor, 1987). The expression of 85B in *M. tuberculosis*-infected monocytes correlates positively with both the amount of secreted TNF-α and subsequent intracellular mycobacterial growth (Wilkinson et al., 2001). Antigen 85B mRNA in both broth cultures and sputum specimens correlates well with the yield of viable *M. tuberculosis* (Desjardin et al., 1999; Hellyer et al., 1999).

Modern laboratory diagnosis of tuberculosis:

The emergence of multidrug-resistant *M. tuberculosis* strains, and increasing co-infection with HIV has fuelled the current pandemic and global resurgence in tuberculosis (De Cock and Chaisson, 1999; Drobniewski et al., 2003). Early diagnosis of tuberculosis and drug resistance improves survival and by identifying infectious cases, promotes contact tracing, implementation of institutional cross-infection procedures, and other public health actions (Drobniewski et al., 2003).

(A) Active disease:

Patients with persistent cough (eg., lasting longer than 2 weeks) should be assessed for tuberculosis (WHO, 2003a). Other common symptoms include fever, night sweats, weight loss, shortness of breath, haemoptysis and chest pain (American Thoracic Society, 2000).

(1) Microscopy:

Introduced by Robert Koch in 1882, microscopy remains a cornerstone of tuberculosis control because it identifies sputum-smear-positive (most infectious) cases (Drobniewski et al., 2003). It is rapid and inexpensive, although it has limited specificity: the smear is positive in only 50–80% of individuals with culture-confirmed primary tuberculosis (Grzybowski et al., 1975; Frieden et al., 2003). Fluorescent staining methods offer higher specimen throughput and possibly greater sensitivity (Drobniewski et al., 2003).

(2) Culture:

Culture is required for definite diagnosis and is essential for drug-susceptibility testing. Rapid automated continuous mycobacterial liquid culture systems should be used in addition to solid culture (eg., the egg-based Lowenstein-Jensen medium or the agar-based Middlebrook formulations, 7H10 or 7H11). Solid culture media allows examination of colony morphology and the identification of mixed cultures, whereas liquid culture enables more rapid diagnosis (Frieden et al., 2003). This is because growth generally occurs within 7-21 days in liquid media (Morgan et al., 1983). Solid media allows prolonged and inexpensive incubation of cultures, with growth occurring in 6 weeks or longer. Positive cultures from these systems are analyzed using DNA

hybridization or comparable systems for the presence/identification of *M. tuberculosis* (Drobniewski et al., 2003).

(3) Radiographic findings:

Findings indicative of tuberculosis include upper-lobe infiltrates, cavity infiltrates, and hilar or paratracheal adenopathy. However, radiographic findings may be subtle or difficult to interpret in HIV-infected patients (Perlman et al., 1997; Frieden et al., 2003).

(B) Latent infection:

The condition of clinical latency in the presence of infection with live *M. tuberculosis* is described as latent tuberculosis infection (Drobniewski et al., 2003). This is demonstrated by a positive reaction to a tuberculin skin test.

(1) Tuberculin skin test (TST):

The intradermal administration of tuberculin has been used as a diagnostic test for tuberculosis infection since the early nineteenth century (Von Pirquet, 1909); the more consistent form of tuberculin, standardized purified protein derivative (PPD-S), has been used to assess latent *M. tuberculosis* infection since 1939 (Seibert and Glen, 1941; Lee and Holzman, 2002). The TST attempts to measure cell-mediated immunity in the form of a delayed-type hypersensitivity response to PPD, which is a crude mixture of antigens, many of which are shared among *M. tuberculosis*, *M. bovis* BCG and several non-tuberculous mycobacteria. Thus, its limitations include low sensitivity in immunocompromised patients (with depressed immunity eg., AIDS patients), cross-reactivity with bacille Calmette-Guerin (BCG) vaccine and a requirement that patients must return 48–72 hours after the test is done to have the result read by measuring the induration (Huebner et al., 1993).

(2) Interferon-γ release assay (IGRA):

This is a whole blood cellular immune-based test that quantifies IFN-γ response. IGRA is based on the principle that T cells of individuals sensitized with tuberculosis antigens produce IFN-γ when they re-encounter mycobacterial antigens (Andersen et al., 2000). A level of IFN-γ production therefore, is presumed to be indicative of tuberculosis infection. An enzyme-linked immunospot (ELISPOT) assay,

which measures IFN-γ production from whole blood or peripheral blood mononuclear cells (PBMC) stimulated with either PPD or more specific antigens has shown promise as a test for latency (Lalvani et al., 2001; Doherty et al., 2002). IGRAs have several advantages over the TST (Lalvani, 2003; Barnes, 2004). Because the test is done *in vitro* and does not involve measurements such as skin induration, the results are less subjective, and a single visit by the patient is adequate. Newer assays that use *M. tuberculosis*-specific region of difference 1 (RD1) antigens may have advantages over the PPD-based TST, in terms of higher specificity (Andersen et al., 2000; Lalvani, 2003), better correlation with exposure to *M. tuberculosis,* and less cross-reactivity due to BCG vaccination and non-tuberculous mycobacterial infection.

Treatment and control:

The goals of treatment are to ensure cure without relapse, to prevent death, to stop transmission, and to prevent the emergence of drug resistance (Frieden et al., 2003). To control tuberculosis, WHO and IUATLD recommend the DOTS (WHO, 2002), which has five elements: political commitment, diagnosis primarily by sputum-smear microscopy among patients attending health facilities, short-course treatment with effective case management (i.e., direct observation), regular drug supply, and systematic monitoring to assess outcome of every patient started on treatment. The international targets for tuberculosis control by 2005 are to detect 70% of new pulmonary smear-positive cases annually, and to treat 85% of detected cases successfully (WHO, 2000).

Tumor necrosis factor-alpha (TNF-α):

TNF-α was first discovered in 1975 as a macrophage-derived molecule that induced a haemorrhagic necrosis of tumors transplanted subcutaneously in mice (Carswell et al., 1975). It is a member of the TNF superfamily of cytokines, consisting mainly of homotrimeric proteins involved with immune regulation and inflammation that includes lymphotoxin-α (TNF-β), CD40 ligand and Fas ligand (Darnay and Aggarwal, 1999). TNF-α is a pleiotropic cytokine that plays a central role in inflammation and apoptosis (Sedgwick et al., 2000; MacEwan, 2002). It is synthesized as a 26 kDa, type II transmembrane protein that is 233 amino acids in length (Pennica

et al., 1984; MacEwan, 2002). It contains a 30 amino acids cytoplasmic domain, a 26 amino acids transmembrane segment, and a 177 amino acids extracellular region (Wang et al., 1985; Ishisaka et al., 1999). TNF-α is assembled intracellularly to form a transmembrane, non-covalently-linked homotrimeric protein. The 157 amino acids residue soluble form of TNF-α (sTNF-α) is released from the C-terminus of the transmembrane protein through the activity of TNF-α-converting enzyme (TACE), a membrane-bound disintegrin metalloproteinase (Moss et al., 1997; Kriegler et al., 1988).

The granulomatous immune response is characterized by delayed hypersensitivity and is mediated by various cytokines released by the stimulated mononuclear phagocytes, including TNF-α (Morimoto et al., 1989; Ellner and Wallis, 1989). TNF-α is a pro-inflammatory cytokine and following appropriate stimulation, many cells produce TNF-α. The main cellular sources of TNF-α during an inflammatory response are monocytes (Frankenberger et al., 1996) and macrophages (Sakurai et al., 1985), but it can also be produced by other cells including NK cells, T and B lymphocytes (Ware et al., 1992), mast cells (Bissonnette et al., 1995), dendritic cells (Zhou et al., 1995), astrocytes (Lee et al., 1993), osteoblasts (Modrowski et al., 1995) and neurons (Tchelingerian et al., 1996). Expression of the TNF-α gene in macrophages can be stimulated by both exogenous and endogenous factors: bacteria, viruses, parasitic organisms, irradiation, trauma, ischemia, cytokines [IL-1β, IL-2, IFN-γ, granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF)] and by TNF-α itself (Larche et al., 2005). Lipopolysaccharide (LPS) and bacterial products are strong stimuli of TNF-α synthesis (Bazzoni and Beutler, 1996). TNF-α is recognized as a significant mediator of macrophage activation for intracellular killing of bacilli following ingestion by phagocytic cells (Wallis et al., 1993). Evidence is accumulating that TNF-α is vital to host defense and antibacterial resistance against infections caused by facultative intracellular organisms, particularly by *M. tuberculosis* which is a potent inducer of cytokine production (Valone et al., 1988; Takashima et al., 1990).

Although this cytokine is involved in multiple cell regulatory and differentiation processes leading to immunity in tuberculous infection, however, TNF-α also mediates effects deleterious to the host contributing to the pathophysiology of tuberculosis. High levels of the cytokine at the site of infection induce an excessive damaging inflammatory response that overwhelms its beneficial effects (Bekker et al., 2000). Therefore, an understanding of the mechanisms that regulate TNF-α expression is important in achieving a harmonious balance between the outcome of both its beneficial and pathologic effects.

(A) Ligand structure and receptors:

The crystal structure of TNF indicates that it is biologically active as a trimer, both as a soluble and as a transmembrane factor (McWhirter et al., 1999; Idriss and Naismith, 2000). The type II transmembrane protein TNF can be considered as the prototype of the TNF cytokine superfamily, a core group of structurally related ligands [encompassing lymphotoxin (LT)α, LTβ, and LIGHT] (Locksley et al., 2001; Granger and Ware, 2001). The TNF cytokine family members interact with more than one receptor of the corresponding superfamily of cognate receptors (Fig. 2). These cytokines and receptors play important roles in the coordinated development of the immune system and the protection from pathogens (Pfeffer, 2003).

Both the homotrimer TNF receptors, TNFR-I (TNFRp55: 55 kDa) and TNFR-II (TNFRp75: 75 kDa) interact with, and are involved in binding and signal transduction to both soluble and transmembrane TNF (Ehlers, 2003). However, the membrane-associated form mostly binds to TNFR-II, whereas soluble TNF preferentially binds to TNFR-I, with greater functional consequences than the former (Papadakis and Targan, 2000). TNFR-I contains a characteristic structural cassette, termed death domain, in its intracytoplasmic sequence that is conserved within a distinct subset of other TNFR family members, such as CD95, DR3, DR4 and DR5 (Tartaglia et al., 1993; Locksley et al., 2001).

(B) TNF-α signal transduction machinery:

TNFR-I has been shown to be essential for surviving infections with intracellular bacteria, such as *M. tuberculosis, Salmonella typhimurium, M. avium,*

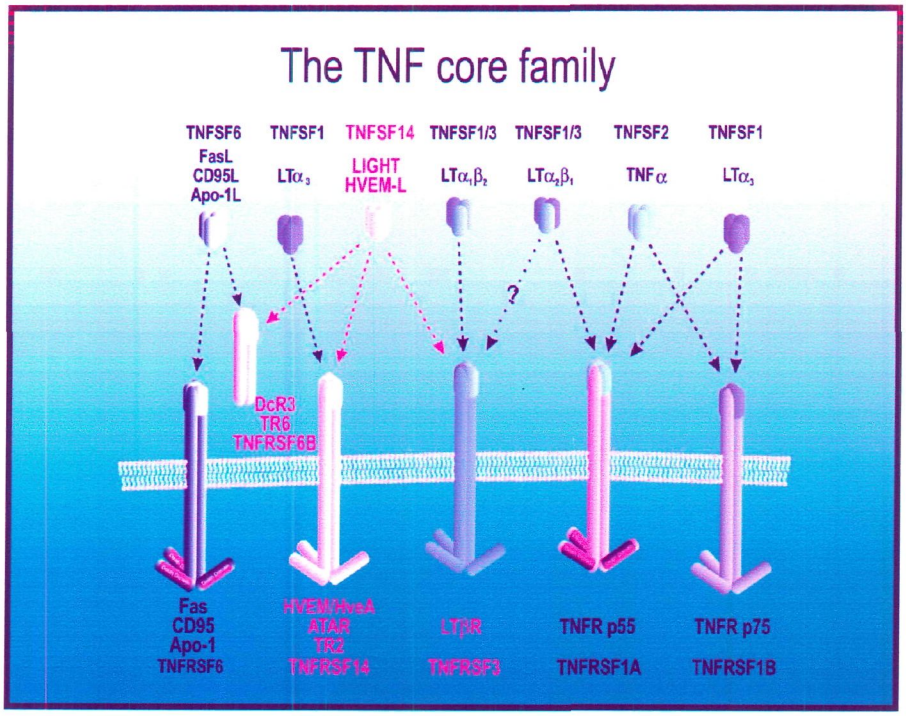


Figure 2. Schematic View of the Members of the Core TNF and TNFR Superfamilies: Arrows show the interaction of the individual cytokines with their cognate receptors. (Source: Klaus Pfeffer. Cytokine & Growth Factor Reviews. 2003 14: 185–191)

Listeria monocytogenes (Pfeffer et al., 1993; Everest et al., 1998; Ehlers et al., 1999). The death domain is required for the signal transduction of the physiological functions of TNFR-I *in vivo* (Plitz et al., 1999). TNF-α exerts its functions by interaction with the death domain-containing TNFR-I and the non-death domain-containing TNFR-II. Important proteins that interact directly or indirectly with the cytoplasmic domains of TNFR-I and TNFR-II are receptor-interacting protein (RIP), a serine/threonine kinase, and TNF receptor-associated factor (TRAF)-1 and -2 (Orlinick and Chao, 1998; Verrecchia and Mauviel, 2004). RIP is required for NF-κB activation in response to TNF-α but not IL-1 or LPS (Kelliher et al., 1998). TRAF-1 and -2 define a novel group of adaptor proteins involved in signal transduction by most members of the TNF receptor family, of IL-1 receptor and IL-17 receptor, as well as some members of the TLRs family (Verrecchia and Mauviel, 2004). TRAF-2 is currently the best characterized TRAF family member, having a key role in mediating TNFR1-induced signalling cascades leading to activation of NF-κB and JNK (c-Jun N-terminal kinase) (Wajant and Scheurich, 2001; Chen and Goeddel, 2002).

(1) NF-κB activation pathway:

NF-κB proteins are a family of cytoplasmic heterodimeric transcription factors that are inactive due to their association with an inhibitory protein IκB (Verrecchia and Mauviel, 2004). Potent activators, such as TNF-α, IL-1, or LPS, activate IKK complex (Bonizzi and Karin, 2004) (Fig. 3). Once activated, IKK induces rapid degradation of the IκBs (especially IκBα) within minutes. For IκBα this degradation process consists of a series of well-characterized steps, which seem to be relevant to the other IκBs (Baldwin, 1996; Ghosh et al., 1998). Inducible IκB phosphorylation, one of the earliest events in the common activation pathway, occurs at serines 32 and 36 in IκBα, and mutation of either serine (even to a threonine) greatly inhibits the degradation process (Traenckner et al., 1995; DiDonato et al., 1996). Phosphorylation leads to the immediate recognition of IκBα by the recently identified F-box/WD40 $E3RS^{IκB}$/β-TrCP (Yaron et al., 1998; Laney and Hochstrasser, 1999; Maniatis, 1999), which consequently results in the polyubiquitinylation of IκBα primarily at lysines 21 and 22 by an SKp1–Cullin–F-box (SCF)-type E3 (Scherer et al., 1995; Baldi et al., 1996). This modification then targets IκBα for rapid degradation by the 26S proteasome.

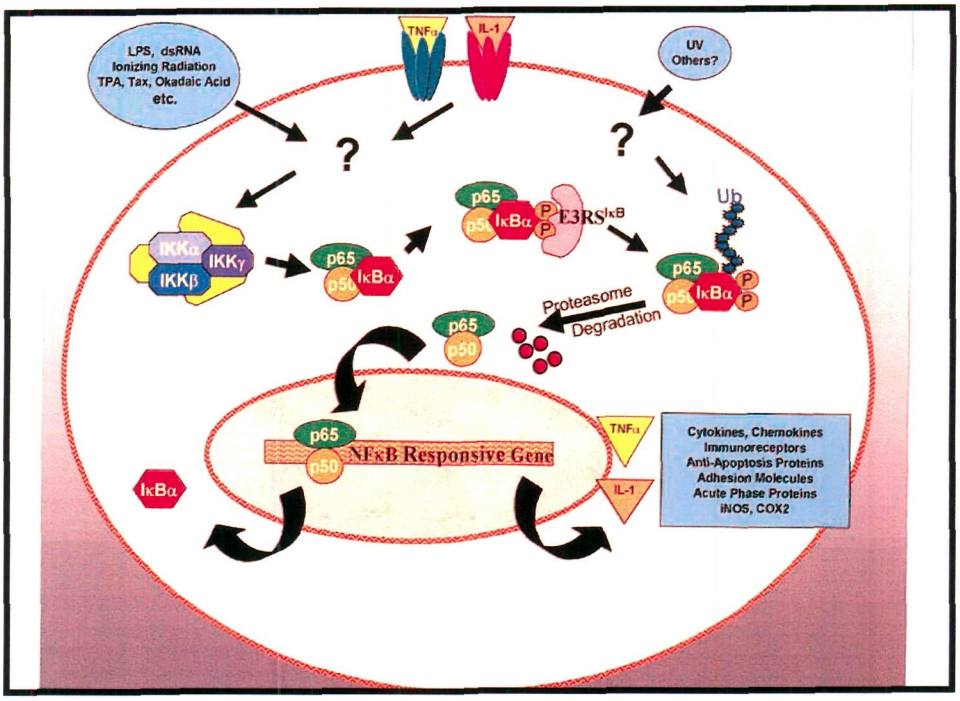


Figure 3. Schematic Model of the NF-κB Activation Pathway.
(Source: Karin and Ben-Neriah. Annual Review of Immunology. 2000 18: 621–663)

The degradation of its inhibitor exposes the nuclear localization sequence (NLS) of NF-κB resulting in binding to karyopherins and translocation of NF-κB to the nucleus (Karin and Ben-Neriah, 2000). It is important that inhibitors of the 26S proteasome efficiently block NF-κB nuclear translocation, indicating that neither phosphorylation nor ubiquitinylation is sufficient to dissociate IκB from NF-κB (DiDonato et al., 1995; Alkalay et al., 1995).

(2) MAP kinase pathway:

JNK is typically activated by a variety of physical and chemical stresses, but also by cytokines like TNF-α. JNK can be triggered by two members of the MAPK kinase (MKK): MKK4 and MKK7, which are targets of a variety of MAP3Ks (such as apoptosis signal-regulating kinase: ASK-1) which interact with TRAF-2. Thus, the pathway from TNFR-I to JNK might include TRADD, TRAF-2 and a MAP3K, initiating the kinase cascade leading to JNK and *cJun* (Verrecchia and Mauviel, 2004).

(C) Induction of TNF-α by *Mycobacterium* virulence factors:

TNF-α exhibits both beneficial and pathologic effects, a feature that requires rigorous control of its expression (Yao et al., 1997). Regulation of human TNF-α expression in cells of monocytic lineage is quite complex, involving controls at both transcriptional and post-transcriptional levels (Han et al., 1990). Mycobacterial cell walls contain the polysaccharide LAM, which has a capacity for induction of TNF-α, through CD14 and synthesis at the transcriptional level (Zhang et al., 1993), similar to that of LPS in Gram-negative bacilli (Moreno et al., 1989). There are structural similarities between LAM and LPS. The promoter region of the human TNF-α gene contains a complex array of potential regulatory elements, and studies indicate that maximal LPS induction of the TNF-α promotor is mediated by concerted participation of at least two separate *cis*-acting regulatory elements (Yao et al., 1997). LAM has been considered an important virulence factor of *M. tuberculosis* with ability to downregulate IFN-γ-inducible genes in macrophages, inhibit protein-antigen processing by antigen-presenting cells, scavenge superoxide anion, and decrease protein kinase C activity (Sibley et al., 1990; Chan et al., 1991). Proteins with the

capacity to induce monocyte cytokine production, such as the 58 kDa protein may also be particularly immunogenic (Wallis et al., 1993).

(D) Role of TNF-α in tuberculosis:

TNF-α is a pleiotropic cytokine, and its role in harmful or beneficial inflammatory processes is complex (Keane, 2005). Regulatory processes, influenced by TNF-α, supporting a beneficial outcome for the host are the following: TNF-α has been shown to modify the endothelium and induce chemokine expression, thereby facilitating extravassation of monocytes and T cells from the blood and directing this migration of cells to the infected site (Ming et al., 1987; Wuyts et al., 1998). Thus, TNF-α with its ability to regulate the expression of chemokines, chemokine receptors, and adhesion molecules, is a potent modulator of cell migration, which is important for granuloma formation (Sedgwick et al., 2000; Mohan et al., 2001). TNF-α also acts as a macrophage-activating factor in mycobacterial infection, facilitating intracellular killing of bacilli following ingestion by phagocytic cells (Bermudez et al., 1990). In addition, TNF-α has a role in apoptosis of macrophages infected with *M. tuberculosis* (Keane et al., 2000), mediating macrophage apoptosis, which can kill the infecting bacillus (Keane et al., 2002). TNF-α production is a requirement for formation of granulomas, which sequester mycobacteria and prevent their dissemination. TNF-α is necessary for the accumulation and organization of monocytes, macrophages, and lymphocytes into well-differentiated granulomas (Kindler et al., 1989).

However, in addition to these protective effects, TNF-α also has detrimental pathogenic effects to the host, such as damaging inflammation leading to tissue necrosis (Rothstein and Schrieber, 1988). Excessive production of TNF-α and increased tissue sensitivity to the cytokine have been implicated in the immunopathology of tuberculosis, such as caseous necrotising reactions, which promote the replication and dissemination of the bacteria (Gardam et al., 2003). TNF-α may also mediate induction of HIV expression in latently infected cells in HIV co-infected tuberculosis patients (Fauci, 1991). TNF-α is also an important mediator of systemic inflammation, clinically manifested by fever and wasting. Depending on the production, TNF-α thus promotes containment or dissemination of *M. tuberculosis* and

can contribute to both immune protection and pathology (Rook and Hernandez, 1996; Kaplan and Freedman, 1996).

(E) TNF-α blockade: Anti-TNF-α agents:

Attenuation of the biological activity of TNF has lately become an important therapeutic intervention in the management of a wide variety of chronic inflammatory diseases (Shanahan and St Clair, 2002). Currently two major protocols are being used for the treatment of various chronic inflammatory diseases: Infliximab, an anti-TNF-α neutralizing antibody, and Etanercept, a recombinant molecule made up of two identical chains of the human 75 kDa TNF receptor II fused to the Fc portion of human IgG1 (Gardam et al., 2003).

Infliximab is a human-murine (25% murine) chimeric monoclonal antibody with high binding affinity (Bekker et al., 2000) and specificity for TNF-α (Knight et al., 1993). It forms stable complexes with the monomeric and trimeric forms of soluble TNF-α and with the transmembrane forms of TNF-α (Scallon et al., 2002). It also has the ability to cross-link TNF-α molecules. Binding to transmembrane TNF-α results in macrophage and monocyte lysis by cytotoxicity that depends on antibodies and complement (Lugering et al., 2001). Infliximab does not bind to related cytokines, such as TNF-β, which is involved in the Th1 response (Falcone et al., 1994).

There are subtle differences in the mechanisms of action of these two agents; in particular, drug-mediated apoptosis and monocytopenia appear to be unique to infliximab (Gardam et al., 2003). Infliximab binds more avidly than etanercept does to transmembrane TNF-α and form a more stable complex. More infliximab than etanercept binds to transmembrane TNF-α. Infliximab is more effective at inhibiting transmembrane TNF-α-mediated activation of endothelial cells. Infliximab binds both the monomeric and trimeric form of soluble TNF-α, whereas etanercept effectively binds only to the trimeric form. Etanercept-TNF-α complexes are unstable, resulting in the release of soluble TNF-α. On the basis of these differences, infliximab might be predicted to have a more significant effect on the host's ability to suppress *M. tuberculosis* infection (Gardam et al., 2003).

Drawback of current treatment and control regimens:

There is a growing body of clinical evidence that neutralization of TNF-α is associated with an increased risk of opportunistic infections, including mycobacterial diseases (Dinarello, 2003). A major side effect of anti-TNF-α therapy is the increased risk of developing tuberculosis (Gardam et al., 2003). TNF-blockade-associated tuberculosis shows granuloma disorganization, which may be deficient in restricting inflammation to a localized environment. This could lead to extension of the inflammatory response into otherwise unaffected lung tissues, resulting in the aberrant pathology observed in tuberculous patients treated with TNF blockade (Mohan et al., 2001). Treatment with anti-TNF-α antibodies readily leads to tuberculosis reactivation in patients with rheumatoid arthritis (Maini et al., 1999).

In addition, drug-induced hepatotoxicity, a leading cause of liver injury, is an important and commonly encountered adverse effect with anti-tuberculosis treatment with three potential hepatotoxic drugs: isoniazid, rifampicin and pyrazinamide (Sharma, 2004). Pyrazinamide was found to significantly contribute to the development of hepatotoxicity when given along with isoniazid and rifampicin (Yee et al., 2003). The use or misuse of these drugs over the years has led to an increasing prevalence of MDR strains, establishing an urgent need to develop new effective agents (Cox et al., 2003). MDR-tuberculosis is defined as the disease due to tuberculosis bacilli resistant to atleast isoniazid and rifampicin, the two most powerful anti-tuberculosis drugs (Girard et al., 2005).

The current vaccine BCG is an attenuated strain of *M. bovis*, which primarily causes bovine disease and is a close relative of *M. tuberculosis*. This strain was generated in the early 1920s by the French researches Albert Calmette and Cannille Guerin by cultivation on solid medium containing bile (Kaufmann, 2005). Most studies have demonstrated that BCG vaccines afford a higher degree of protection against severe forms of tuberculosis, such as meningitis and disseminated tuberculosis, than against moderate forms of the disease (Girard et al., 2005). Even with high coverage, BCG has not had any substantial effect on transmission or incidence, because its main action is to prevent serious (but infectious) disease in children (Styblo, 1991). Adverse events from BCG vaccination can occur, including local

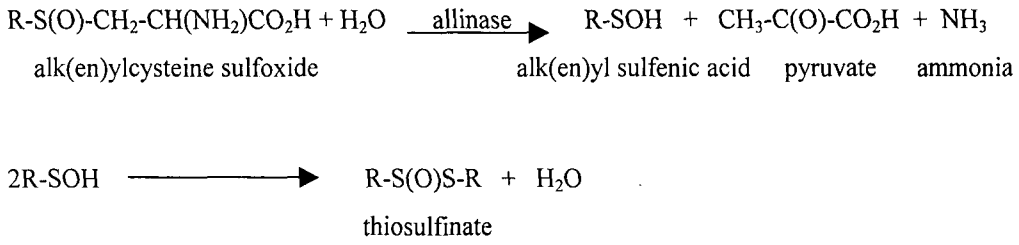
subcutaneous abscess and ulcers, suppurative lymphadenitis, and, more rarely, disseminated disease (Lotte et al., 1988). In addition, BCG vaccination may only provide protection against primary infection and be of little help in already infected individuals or in cases of reactivation tuberculosis (Smith and Starke, 2004). Despite continuing efforts to develop more effective tuberculosis vaccines, none have been identified to date. Even if one were to be developed, it might not prevent progression to active disease among the more than two billion people already infected with *M. tuberculosis*. Therefore, even if a new vaccine were to be implemented worldwide, more effective treatment systems would be required for decades (Frieden et al., 2003).

Natural products in tuberculosis prevention – Garlic and allicin:

Garlic (*Allium sativum)* is well known for its medicinal benefits and has been used in herbal medicine for thousands of years (Juszkiewicz et al., 2004). It has been cultivated since ancient times and used as a food, spice/condiment and folk remedy for many centuries (Rassoula et al., 2005). Garlic has a wide spectrum of pleiotropic actions, including antibacterial, antifungal and antioxidant activity. It is a strong antibacterial agent and acts as an inhibitor of both Gram-positive and Gram-negative bacteria (Ankri and Mirelman, 1999; Juszkiewicz et al., 2004). It also has beneficial effects on the cardiovascular and immune systems (Harris et al., 2001). During the past years, there has been a growing awareness of the potential medicinal uses of garlic. The antioxidant properties of garlic are well documented (Gedik et al., 2005; Sener et al., 2005). Garlic has been related to various vasoprotective and anti-inflammatory effects (Borek, 2001; Sela et al., 2004). Garlic extracts can modulate the IL-1α-mediated cell response (adhesion) of human endothelial cells (Rassoula et al., 2005). Adhesion of mononuclear monocytes to endothelial cells and trans-endothelial migration of monocytes mediated by cell adhesion molecules (CAMs) are important steps in both inflammation and in the atherosclerotic process (Lkeda et al., 1998; Richter et al., 2003). Antioxidants act at the cellular level to downregulate cytokine-stimulated CAM expression in endothelial cells with a subsequent decrease in leukocyte adhesion (Zapolska-Downar et al., 2001). Since several components of garlic are known to possess antioxidant properties (Borek, 2001), garlic extract may be useful in reducing oxidative stress. Garlic oils stimulate the activity of glutathione

peroxidase and inhibit the decreased ratio of reduced to oxidized glutathione produced by 12-*O*-tetradecanoylphorbol-13-acetate in epidermal cells (Perchellet et al., 1986). In addition, garlic extract was identified as a potent inhibitor of leukocyte migration (Hofbauer et al., 2001).

Ninety-five percent of the sulphur in intact garlic cloves is found in two classes of compounds in similar abundance: the S-alkylcysteine sulfoxides and γ-glutamyl-S-alylcysteines (Lawson, 1998). Upon cutting or crushing garlic cloves, odourless cysteine sulfoxides are very rapidly converted to a new class of compounds, the thiosulfinates, which are responsible for the odour of freshly chopped garlic. The most abundant sulphur compound in garlic is alliin (S-allylcysteine sulfoxide) and the main thiosulfinate formed upon crushing garlic is allicin. The formation of thiosulfinates takes place when the cysteine sulfoxides, which are located only in the clove mesophyll storage cells, come in contact with the enzyme allinase or alliin lyase (E.C. 4.4.1.4.), which is located only in the vascular bundle sheath cells (Juszkiewicz et al., 2004; Chaverri et al., 2005):



At the initial period of this reaction, pyruvate, ammonia and alk(en)yl sulfenic acid RS(O)H are formed; the latter undergoes rapid condensation to form thiosulfinates R-S(O)S-R, where R represents: methyl, 1-propenyl or 2-propenyl group (Juszkiewicz et al., 2004).

Allicin (diallyl thiosulfinate) is the major biologically active component and thiosulfinate compound of freshly crushed garlic (chemical structure shown in Fig. 4). Garlic-rich organosulphur compounds and their precursors (allicin, diallyl sulfide and diallyl trisulfide) are believed to play a key role in the biological effects of garlic

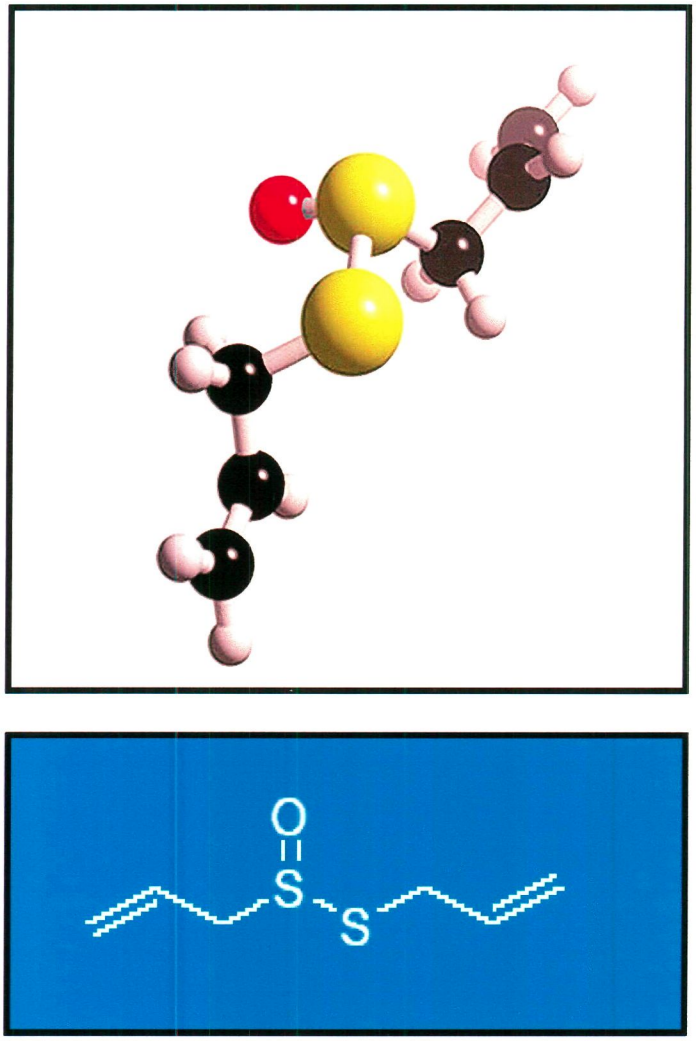


Figure 4. Structures of Allicin

(Source: www.3dchem.com/molecules.asp?ID=156)

(Ankri and Mirelman, 1999). Garlic extract and S-allyl cysteine reduce hydrogen peroxide or TNF-induced NF-κB activation (Ide and Lau, 2001). Allicin was reported to affect different biological activities such as antimicrobial, antiparasitic and antifungal activities (Ross et al., 2001). Allicin has radical scavenging properties in activated granulocytes (Siegers et al., 1999) and may also inhibit inducible nitric oxide synthase expression in activated macrophages (Dirsch et al., 1998). The antimicrobial activity of allicin is considered to depend on its inhibitory effects on certain thiol-containing enzymes via strong SH-modifying properties, as reflected by the production of S-allylmercaptocysteine (Ankri and Mirelman, 1999; Rabinkov et al., 2000). Allicin freely permeates into or across phospholipid bilayers and interacts with the thiol groups of intracellular molecules (Miron et al., 2000). L-Cysteine of the antioxidant glutathione may also be a target of allicin (Rabinkov et al., 2000). It has been found that allicin scavenges $OH^{\cdot}$ and inhibits lipid peroxidation (Prasad et al., 1995). Higher doses (>100 μM) of allicin have proven toxic to mammalian cells. At present a variety of biological effects of allicin are attributed both to its SH-modifying and its antioxidant activity (Ankri et al., 1997). Although allicin, the active component of freshly crushed garlic, has different biological activities, however its anti-inflammatory properties warrant further investigation. Thus, we studied the incorporation of this herbal component as an anti-inflammatory agent for tuberculosis management.

Objectives of the present study:

The human immune response to *M. tuberculosis* is not well characterized. To better understand the cellular immune response to tuberculosis, in the present study, a human monocyte culture system infected with *M. tuberculosis* at an infective ratio of 1:1 (bacteria:cell) was employed. Here we have examined the interaction of virulent *M. tuberculosis* (H37Rv) with human monocytes, focussing on the role of TNF-α and *M. tuberculosis* 85B. In this investigation, a systematic study was carried out to characterize the sera and monocytes of tuberculosis patients with respect to TNF-α and *M. tuberculosis* 85B by real-time RT-PCR, direct binding ELISA and competition ELISA. The modulatory effects of the natural antioxidant allicin on *M. tuberculosis*

85B expression in H37Rv cultures were evaluated by real-time RT-PCR, SDS-PAGE, gel retardation assay, direct binding ELISA and competition ELISA. The interaction of *M. tuberculosis* 85B and TNF-α after 24 hours of infection of human monocytes with *M. tuberculosis* was investigated by RT-PCR and real-time RT-PCR. To fully delineate the contribution of the innate immune response to the pathogenesis of *M. tuberculosis* infection, the roles played by ROIs and RNIs, induction of TNF-α, activation of NF-κB and monocyte activation, in the expression of *M. tuberculosis* 85B were examined.

Toxicity assessment of allicin on viability of human monocytes was assessed by cell viability assays like MTT and trypan blue exclusion assay as well as by assessing human housekeeping genes like β-actin and 18S rRNA by RT-PCR and real-time RT-PCR, respectively. The effects of allicin on expression of TNF-α and *M. tuberculosis* 85B in *M. tuberculosis*-infected monocytes after 24 hours of infection were probed by RT-PCR, real-time RT-PCR and ELISA. The repertoire of TNF-α, TNFR-I and TNFR-II in supernatants of *M. tuberculosis*-infected monocyte cultures that were treated and untreated with allicin was assessed by commercially available ELISA kits. Glutathione peroxidase activity was also evaluated in *M. tuberculosis*-infected monocytes after 24 hours of infection in the presence or absence of allicin.

Experimental

Materials:

Recombinant human (rh) TNF-α, monoclonal anti-TNF-α antibody, soluble TNFR-I, soluble TNFR-II, N-acetyl-cysteine, H_2O_2, SN50 (an inhibitor of NF-κB) and its analogue SN50/M, oxidized ATP, N^G-monomethyl-L-arginine-monoacetate, nonoate-9, sodium nitroprusside, agarose, bovine serum albumin, Coomassie Brilliant Blue G-250 and R-250, p-nitrophenyl phosphate, anti-human IgG alkaline phosphate conjugate, Tween-20, dithiothreiotol, phenylmethylsulphonyl fluoride, standard protein markers, protein A sepharose CL-4B, sodium azide, ethidium bromide, chloroform, isoamyl alcohol, reduced glutathione, glutathione reductase, cumene hydroperoxide and reduced β-nicotinamide adenine dinucleotide phosphate were from Sigma Chemical Company, U.S.A. SN50 is a hybrid peptide containing nuclear localization sequence of p50 subunit of NF-κB heterodimer and has been shown to completely inhibit the translocation of NF-κB in human cell lines at 100 μg/ml (Lin et al., 1995).

Allicin was obtained from LKT Laboratories, Inc. U.S.A. Ficoll-Paque was from Pharmacia, Piscataway, NJ, U.S.A. Middlebrook 7H9 broth, Middlebrook ADC enrichment fluid, RPMI-1640 medium were from HiMedia, India. MTT cell viability assay kit and immunoassay kits for TNF-α and its receptors were from R & D Systems, U.S.A. 12-wells tissue culture plates were obtained from Techno Plastic Products (TPP), Switzerland. Polystyrene microtitre flat bottom ELISA plates having 96 wells (7 mm diameter) were from NUNC, Denmark. All other chemicals were of the highest analytical grade available.

Study subjects:

Venous blood was obtained from healthy nonsmoking adult volunteers with no history of tuberculosis or positive tuberculin skin test. Also, blood from patients with active pulmonary tuberculosis was obtained from the patients admitted to the J.N. Medical College Hospital of A.M.U. The diagnosis of tuberculosis was based on demonstration of acid-fast bacilli on sputum smear and later confirmed by positive culture of *M. tuberculosis*. Serum was separated and stored at –20°C until required.

Determination of protein concentration:

Protein was estimated by the methods of Lowry et al. (1951) and Bradford (1976).

(A) Protein estimation by the Lowry (Folin-Ciocalteau) method:

Protein estimation by this method involves complexing of the protein's peptide bonds with Cu^{2+} under alkaline conditions (Lowry et al., 1951). The resultant Cu^{+} appears to catalyze the oxidation of tyrosine and tryptophan residues by reducing phosphomolybdotungstate anions in the Folin reagent (a mixture of sodium tungstate, molybdate and phosphate), added subsequently. This reaction develops a blue colour due to the formation of heteropolymolybdenum blue, which can be quantified by its absorbance at 660 nm.

Reagents:

(i) Folin-Ciocalteau reagent

The reagent was diluted 1:4 with distilled water before use.

(ii) Alkaline copper reagent

The components of alkaline copper reagent were prepared as follows:

(a) 2% sodium carbonate in 100 mM NaOH

(b) 0.5% copper sulphate in 1% sodium potassium tartarate

The working reagent was prepared fresh before use by mixing the two components in the ratio 50:1, respectively.

Procedure:

To 1.0 ml of protein sample was added 5.0 ml of freshly prepared alkaline copper reagent. After thorough mixing, the reaction mixture was allowed to stand at room temperature for 10 minutes, followed by the addition of 1.0 ml of 1:4 times diluted Folin-Ciocalteau reagent. The contents were mixed immediately. The reaction was allowed to proceed for 30 minutes at room temperature and each tube was subsequently monitored at 660 nm. The protein content of the unknown sample was determined by using bovine serum albumin to construct a standard calibration curve.

(B) Protein estimation by the Bradford method:

This method is based on strong binding of the dye Coomassie Brilliant Blue G-250, in acidic medium, to protein hydrophobically and at positively charged groups (Bradford, 1976). In the environment of these positively charged groups, protonation is suppressed and a blue color develops (λ_{max}-595 nm).

Preparation of dye:

100 mg of Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol and 100 ml of 85% (v/v) orthophosphoric acid was added. The resulting solution was diluted to a final volume of 1.0 litre and filtered through Whatman No. 1 filter paper to remove undissolved particles.

Procedure:

To 1.0 ml of solution containing 10–100 μg protein was added 5.0 ml of dye solution. The contents were mixed thoroughly by vortexing. The absorbance was read at 595 nm after 5 minutes against a reagent blank.

Polyacrylamide gel electrophoresis of proteins:

Polyacrylamide gel electrophoresis was performed under denaturing conditions as described by Laemmli (1970).

Reagents:

(i) Acrylamide-bisacrylamide (30:0.8)

A stock solution of 30% acrylamide containing 0.8% bisacrylamide was prepared by dissolving 30 gm of acrylamide and 0.8 gm of bisacrylamide in a total volume of 100 ml. The solution was stored at 4°C in an amber coloured bottle.

(ii) Resolving gel buffer

A stock solution was prepared by dissolving 36.3 gm Tris base in 48 ml of 1 N HCl. The contents were mixed, pH adjusted to 8.8 and the final volume brought to 100 ml with distilled water.

(iii) Stacking gel buffer

6.05 gm Tris was dissolved in 40 ml distilled water, pH adjusted to 6.8 with 1 N HCl and the final volume adjusted to 100 ml with distilled water.

(iv) Electrode buffer

3.03 gm Tris, 14.4 gm glycine and 1.0 gm SDS were dissolved in distilled water, pH adjusted to 8.3 and the final volume made up to 1.0 litre with distilled water.

(v) Sample buffer

(a) 6.0 gm of Tris was dissolved in 80 ml distilled water and pH adjusted to 6.8 with phosphoric acid. The final volume was brought to 100 ml with distilled water.

(b) 1.0 mg of bromophenol blue and 12.5 ml of glycerol were added to 12.5 ml of the above solution. β-mercaptoethanol was added just before use.

Recipe for 10–20% Gradient Gel

Resolving Gel (total volume: 30 ml)

Reagents	**10%**	**20%**
Acrylamide-bisacrylamide (30:0.8)	5.0 ml	10 ml
Resolving gel buffer	3.8 ml	3.8 ml
10% SDS	150 µl	150 µl
10% Ammonium persulphate	50 µl	50 µl
TEMED	10 µl	10 µl

The final volume was raised to 15 ml each with distilled water.

2.5% Stacking Gel (total volume: 10 ml)

Acrylamide-bisacrylamide (30:0.8)	0.8 ml
Stacking gel buffer	2.5 ml
10% SDS	100 µl
10% Ammonium persulphate	75 µl
TEMED	25 µl

The final volume was raised to 10 ml with distilled water.

Recipe for 7.5% SDS-PAGE (total volume: 10 ml)

Acrylamide-bisacrylamide (30:0.8)	2.5 ml
Resolving gel buffer	2.5 ml
10% SDS	100 µl
10% Ammonium persulphate	50 µl
TEMED	10 µl

The final volume was raised to 10 ml with distilled water.

Procedure:

The glass plates (18 cm x 16 cm) were soaked in chromic acid and thoroughly washed with tap water followed by a final rinse with distilled water and ethanol. The plates were dried and sealed with 1% agarose and 1.5 mm thick spacers. The reagents were mixed and poured between the glass plates. The resolving gel was allowed to polymerize at room temperature, following which, the stacking gel was layered on top. A well-forming comb was inserted immediately and the gel was left to polymerize at room temperature. In case of gradient gels, a gradient of resolving gel was formed with the help of a gradient former (Bio-Rad, model 385). After ensuring complete polymerization, the protein samples (25–100 µg) in one-fourth volume of sample buffer were electrophoresed at 80 volts at room temperature. The gels were stained using 0.25% Coomassie Brilliant Blue R-250 or with silver stain reagent.

Silver staining:

Silver staining was done by the method of Merril et al. (1983). Briefly, the gel was incubated in 40% methanol and 12% acetic acid for 45 minutes followed by incubation in 50% ethanol for 30 minutes. Next the gel was treated with 0.02% hypo (sodium thiosulphate) for 1 minute. After washing with distilled water, the gel was placed in 0.2% silver nitrate (with 0.05% v/v formaldehyde), washed again with distilled water, and transferred to a 6% solution of sodium carbonate (with 0.05% v/v formaldehyde). After colour development, the gel was washed with distilled water and the reaction was arrested by treating the gel with 3% v/v acetic acid and 5% v/v methanol. All the reagents used in this procedure were freshly prepared.

Agarose gel electrophoresis:

Agarose gel was prepared by bringing 2% agarose to molten state in electrophoresis buffer (0.04 M Tris acetate, pH 8.0 containing 0.002 M EDTA). Molten agarose was poured on the gel tray and allowed to solidify for 1 hour at room temperature. The nucleic acid samples in one-tenth volume of stop-mix (30% ficoll, 0.025% xylene cyanole FF and 500 mM EDTA in 10X TAE buffer) were electropheresed for 2–4 hours at 30 mA in the same buffer. The gel was stained with ethidium bromide (0.5 μg/ml).

Isolation of IgG by affinity chromatography:

Protein A sepharose CL-4B was employed as the affinity matrix. It was swelled in 10 mM PBS, pH 7.4 at room temperature for 12 hours. The swelled matrix was washed with PBS and packed in a column having a dimension of 0.9 cm x 5 cm. The packed column was washed with 0.1 M sodium citrate, pH 3.0 in order to elute any bound material. This was followed by equilibration of the packed matrix with 5 volumes of PBS, pH 7.4. Serum diluted with equal volume of PBS, pH 7.4 was loaded onto the column and allowed to bind at a flow rate of 20 ml/hour. Unbound protein was eliminated with PBS and absorbance of the effluent monitored till a negative absorbance was obtained at 280 nm. The bound IgG was eluted with 0.58% acetic acid in 0.85% sodium chloride. To prevent the effect of acidic elution buffer on IgG, fractions were collected in 1 M Tris-HCl, pH 8.5. The fractions containing IgG were monitored at 280 nm. The IgG concentration was determined considering 1.4 OD_{280} = 1.0 mg IgG/ml. The isolated IgG was dialyzed against 10 mM PBS, pH 7.4 and stored at –20°C with 0.1% sodium azide. To check the purity of IgG, the samples were subjected to 7.5% SDS-PAGE.

Gel retardation assay:

The binding of TB-IgG with *M. tuberculosis* sonic extract proteins as well as with MTCF proteins was analyzed by altered electrophoretic mobility on SDS-PAGE under non-reducing conditions. *M. tuberculosis* proteins were allowed to interact with TB-IgG for 2 hours at 37°C and overnight at 4°C. This resulted in the formation of immune complex (IC). Thereafter, the complex was electrophoresed on 7% SDS-PAGE

under non-reducing conditions for 2 hours at 80 V. The electrophoresed gels were visualized by staining with 0.25% Coomassie Brilliant Blue R-250.

Enzyme-linked immunosorbent assay (ELISA):

Antibodies were detected and quantified by ELISA using polystyrene flat-bottom microtitre plates as solid phase. The method described by Alam and Ali (1992), Islam and Ali (1998) was followed for the assay.

Buffers and reagents:

(i) Tris-buffered saline (TBS)
10 mM Tris, 150 mM NaCl, pH 7.4

(ii) Tris-buffered saline Tween-20 (TBS-T)
20 mM Tris, 144 mM NaCl, 2.68 mM KCl and 1.0 ml/litre Tween-20, pH 7.4

(iii) Bicarbonate buffer
15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6

(iv) Substrate buffer (for anti-human IgG alkaline phosphatase conjugate)
15 mM sodium carbonate, 35 mM sodium bicarbonate and 2 mM magnesium chloride, pH 9.6
Substrate: 0.5 mg/ml of p-nitrophenyl phosphate

(A) Direct binding ELISA:

Polystyrene microtitre plates were incubated with 100 µl of protein antigen (30 µg/ml in carbonate/bicarbonate buffer, pH 9.6) for two hours at room temperature followed by overnight incubation at 4°C. The plates were washed thrice with TBS-T and unoccupied sites blocked by 150 µl of BSA (1.5% in TBS, pH 7.4) for 4–6 hours at room temperature. Serially diluted sera in TBS were added to antigen-coated as well as control (antigen uncoated) wells. The antigen-antibody interaction was allowed to proceed for two hours at room temperature followed by overnight incubation at 4°C and subsequently the plates were washed four times with TBS-T in order to remove the unbound antibodies. Bound antibodies were assayed by means of appropriate anti-immunoglobulin alkaline phosphatase conjugate using p-NPP as substrate.

The reaction was stopped with 3 N NaOH and the absorbance of each well was monitored at 405 nm on an ELISA microplate reader. Each sample was coated in duplicate and the results were expressed as a mean of $A_{test} - A_{control}$.

(B) Inhibition ELISA:

The antigen binding specificity of antibody was determined by inhibition experiments (Hasan et al., 1991). Varying concentration of inhibitors (0–20 μg/ml) were mixed with a constant amount of antiserum or IgG. The mixture was incubated for two hours at 37°C followed by overnight incubation at 4°C. The resulting immune complex was employed in the immunoassay instead of serum. The rest of the steps were as in direct binding ELISA. The results were expressed as percent inhibition.

$$\textbf{Percent inhibition} = \left(1 - \frac{A_{inhibited}}{A_{uninhibited}}\right) \times 100$$

Preparation of mycobacteria:

Virulent laboratory-adapted *M. tuberculosis* (H37Rv) were grown in Middlebrook 7H9 broth supplemented with Middlebrook ADC enrichment fluid at 37°C in 5% CO_2. Mycobacterial cultures were harvested at midlogarithmic (14 days) phase. Aliquots of the stock were kept at −70°C until use. The viability of the stock remained >99% at 1 year. Before use, aliquots were defrosted, then vortexed with glass beads for 15 minutes, followed by equilibration at 37°C for 45 minutes. This treatment results in single cell suspension of mycobacteria (Toossi et al., 1996).

Isolation of mycobacterial antigens:

Midlogarithmic mycobacterial cultures (14 days) were heat-killed at 100°C for one hour in a boiling water bath, followed by centrifugation at 10,000 rpm for 30 minutes at 4°C. The culture filtrate containing secreted proteins was separated and stored at –20°C until further use.

(A) Desalting of *M. tuberculosis* culture filtrate (MTCF) proteins:

The supernatant was sterilized by filtration through a 0.22 micron pore size membrane (Millipore Corp., USA). MTCF was concentrated 50 fold by ammonium

sulphate and dialyzed against 10 mM PBS, pH 7.4 for desalting. The protein content was determined by the method of Lowry et al. (1951) against a BSA standard. The culture filtrate preparations were stored in small aliquots at –20°C.

(B) *M. tuberculosis* sonic extract (MTSE) proteins:

Heat-killed bacilli were washed with sterile TBS, pH 7.4 and suspended in sonicating buffer containing 1 mM DTT, 1 mM PMSF, 1.0 µl/ml β-mercaptoethanol and proteinase inhibitors cocktail in TBS, pH 7.4. The cell suspension was disrupted by sonicating at 4°C for 15 minutes in a sonicator and the cell debris was removed by ultra centrifugation at 20,000 rpm for 15 minutes. The protein content was determined by the method of Lowry et al. (1951). The sonic extract preparations were stored at –20°C in small aliquots until required.

Preparation of RPMI-1640 medium:

Dehydrated RPMI-1640 medium of one unit vial (16.3 gm) was suspended in 950 ml of tissue culture-grade water at room temperature with constant, gentle stirring until the medium was completely dissolved. The container was rinsed with tissue culture grade water to remove all traces of powder and added to the above solution. 3.7 gm sodium bicarbonate was added to the medium and stirred until dissolved. The final volume was brought to 1000 ml with tissue culture grade water. The medium was sterilized immediately by filtering through a sterile membrane filter with a porosity of 0.22 micron using positive pressure rather than vacuum to minimize the loss of carbon dioxide, and stored at 4°C till use.

Preparation of PBMC:

Step 1: In order to isolate peripheral blood mononuclear cells (PBMCs), 60 ml of blood was drawn from a healthy volunteer into 60 cm^3 syringes containing 3.8 units heparin/ml. The heparinized blood, in 15 ml aliquots, was transferred to sterile 50 ml polypropylene centrifuge tubes and diluted 1:1 with sterile 10 mM, PBS, pH 7.4 at room temperature, followed by gentle mixing by inverting the tube a few times.

Step 2: Diluted blood was underlayered with 15 ml of Ficoll-Paque at room temperature using an 18 gauge spinal needle. Care was taken to prevent mixing of the layers. The gradient was centrifuged at 1800 rpm for 30 minutes at room temperature with the centrifuge brake turned off.

Step 3: Using a sterile pipette, the upper clear layer containing plasma was removed. The PBMCs appeared as a dense white band (buffy layer) above the red blood cells and granulocytes layer. This was removed with another sterile pipette. The banded cells were combined in 10 ml aliquots.

Step 4: Ten milliliters of banded PBMCs were diluted with 25 ml of PBS in sterile 50 ml polypropylene centrifuge tubes and centrifuged at 1100 rpm for 12 minutes at room temperature to remove platelets, which remain in the supernatant. The PBMC pellets were combined to four tubes, diluted in 30 ml PBS and centrifuged at 1100 rpm for 10 minutes at room temperature. This wash was repeated.

Step 5: The pellets were then combined and resuspended in 30 ml complete medium (CM) (RPMI-1640 medium containing 2 M L-glutamine, 25 mM HEPES, and no antibiotics). An aliquot was diluted 20-fold and counted using a hemocytometer under a light microscope using 10x ocular and 40x objective.

Preparation of autologous serum for monocyte culture:

From the same donor, 30 ml of blood was drawn without anticoagulant and transferred to serum separator tubes. The blood was allowed to clot for at least 30 minutes, then centrifuged at 3000 rpm for 15 minutes at room temperature and the serum filtered through a sterile 0.22 μm filter unit. Autologous serum can be stored for a year or longer at –20°C.

Cell culture:

PBMCs ($5x10^6$ cells/well) were added in 12-wells tissue culture plates in complete RPMI-1640 medium, and were subsequently incubated at 37°C, 5% CO_2 for 1–2 hours for adherence. Thereafter, non-adherent cells were removed by washing the plates extensively 4 times with RPMI-1640 medium. The adherent monocytes were

cultured in RPMI-1640 supplemented with 2% autologous serum, followed by overnight resting at 37°C, 5% CO_2. This population of adherent cells is up to 95% monocytes, as observed by cytostaining and is 99% viable (Toossi et al., 1996). Prior to infection, the plates were washed twice with RPMI-1640 medium.

Infection and co-culture of monocytes with supplements:

Monocytes were infected with *M. tuberculosis* (H37Rv) at 1:1 (bacteria/cell) in 30% autologous unheated serum for 90 minutes at 37°C, 5% CO_2. Subsequent to this, the infected monocytes were washed four times with complete medium. Cells harvested at this time point were considered as time zero after infection (t_0). Other cultures received RPMI-1640 medium with 2% autologous serum. As per experimental design, some cultures, immediately after infection received 10 ng/ml rhTNF-α, 10 ng/ml anti-TNF-α antibodies, varying doses of allicin (0–500 ng/ml) and a mixture of 10 ng/ml rhTNF-α and 500 ng/ml allicin, respectively. Also, some cultures received 10 nM H_2O_2, 10 mM NAC, 100 μg/ml SN50 and SN50/M. Cultures were then harvested after 24 hours and cells were lysed in 0.5 ml of TRIZOL Reagent (Invitrogen Inc. Carlsbad, CA, USA). The cell-free culture supernatants were kept at –70°C.

Treatment with allicin and monocytes viability assay:

The effect of allicin (0–500 ng/ml) on the viability of monocytes was assessed by using MTT Cell Viability Assay Kit (R & D Systems) according to the manufacture's instructions provided.

Reagents supplied in the kit:

Component	Quantity	Storage conditions
MTT reagent	25 ml	2 – 8°C
Detergent reagent	250 ml	18 – 24°C

Assay procedure:

Adherent monocytes infected with *M. tuberculosis* (H37Rv) at 1:1 (bacteria/cell) were gently scraped with RPMI-1640 medium. After this, monocytes (3×10^4 cells/well in 100 μl) were added in 96-well tissue culture plates. Cells were

incubated in RPMI-1640 with 2% autologous serum containing allicin (50, 100, 250, and 500 ng/ml) for 24 hours at 37°C, 5% CO_2. After 24 hours, 10 µl of MTT reagent (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) was added to each well and incubation was continued for an additional two hours. When a purple precipitate was clearly visible under the microscope, 100 µl of detergent reagent was added to all wells, including control wells and incubated for two hours in the dark at 20°C. After incubation, the precipitate was solubilized and the absorbance of the resulting solution was measured at 570 nm using a microplate reader. Control cells were treated exactly the same except that no allicin was added to the wells. The percentage of viable cells was calculated by the formula as described by Islam et al. (2000) and the results are expressed as "Viable cells (% of control cells)".

$$\text{Viable monocytes (\% of control cells)} = \frac{\text{Absorbance value of control cells}}{\text{Absorbance value of treated cells}} \times 100$$

Trypan blue exclusion assay for monocytes viability:

Adherent monocytes were gently scraped with RPMI-1640 medium. Trypan blue suspension (1.6 mg/ml in saline solution) was added to the monocytes at a final concentration of 0.8 mg/ml. The cells were kept at 37°C for 7 minutes in a CO_2 chamber (5%), mounted on a hemocytometer and then observed under light microscope. The cells taking up Trypan blue (dead cells), and cells excluding the dye (viable cells) were counted. Percentage of viable cells was calculated by the following formula:

$$\text{\% Cell viability} = \frac{\text{Total viable cells (unstained)}}{\text{Total cells (stained plus unstained)}} \times 100$$

TNF-α Immunoassay:

The concentration of TNF-α in various culture supernatants as well as in serum of tuberculosis patients was determined by use of a commercial ELISA Kit (R & D Systems). This assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TNF-α was available pre-coated onto a

microplate. Standards and samples were pipetted into the wells and any soluble TNF-α present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TNF- α was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of TNF-α bound in the initial step. The colour development was stopped and the intensity of the colour was measured.

Reagents supplied in the kit:

TNF-α microplate - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against TNF-α.

TNF-α conjugate - 21 ml of polyclonal antibody against TNF-α conjugated to horseradish peroxidase, with preservatives.

TNF-α standard - 10 ng of recombinant human TNF-α in a buffered protein base with preservatives, lyophilized.

Assay diluent RD1F - 6 ml of a buffered protein base with preservatives. It contained a precipitate and was mixed well before and during use.

Calibrator diluent RD6-35 - 21 ml of animal serum with preservatives.

Wash buffer concentrate - 21 ml of a 25-fold concentrated solution of buffered surfactant with preservatives.

Colour reagent A - 12.5 ml of stabilized hydrogen peroxide.

Colour reagent B - 12.5 ml of stabilized chromogen (tetramethylbenzidine).

Stop solution - 6 ml of 2 N sulphuric acid.

Plate covers - 4 adhesive strips.

Working reagents:

Wash buffer - 20 ml of wash buffer concentrate was diluted into deionized or distilled water to prepare 500 ml of wash buffer.

Diluted calibrator diluent RD6-35 - 20 ml of calibrator diluent RD6-35 was mixed with 80 ml of deionized or distilled water to yield 100 ml of diluted calibrator diluent RD6-35.

Substrate solution - Colour reagents A and B were mixed together in equal volumes within 15 minutes of use to form substrate solution. It was protected from light.

TNF-α standard - TNF-α standard was reconstituted with 1.0 ml of distilled water. This reconstitution produced a stock solution of 10,000 pg/ml. The standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Assay procedure:

50 µl of assay diluent RD1F was added to each well of 96 well polystyrene microplate coated with a mouse monoclonal antibody against TNF-α. Thereafter, 200 µl of standards, samples, or control per well was added, covered with the adhesive strip provided and incubated for 2 hours at room temperature. The plate was washed four times by filling each well with wash buffer using a squirt bottle. After washing, 200 µl of TNF-α conjugate was added to each well, covered with a new adhesive strip and incubated for 1 hour for cell culture supernatants and 2 hours for serum samples at room temperature. After four washings with wash buffer, 200 µl of substrate solution was added to each well and incubated for 20 minutes at room temperature in the dark, a blue colour appeared. Thereafter, 50 µl of stop solution was added to each well to stop the reaction. Then the absorbance of each well was determined within 30 minutes, using a microplate reader set to 450 nm. The cut off or lower limit of sensitivity was 4.4 pg/ml.

Soluble TNFR-I and TNFR-II Immunoassay:

The concentrations of soluble TNFR-I and TNFR-II in various culture supernatants were determined by use of commercial ELISA Kits (R & D Systems).

Reagents supplied with TNFR-I kit:

sTNFR-I microplate - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against sTNFR-I.

sTNFR-I conjugate - 21 ml of polyclonal antibody against sTNFR-I conjugated to horseradish peroxidase, with preservatives.

sTNFR-I standard - 2.5 ng of recombinant human sTNFR-I in a buffered protein base with preservatives, lyophilized.

Assay diluent HD1-7 - 6 ml of a buffered protein base with preservatives.

Calibrator diluent RD5-5 - 2 vials (21 ml/vial) of a buffered protein base with preservatives.

Wash buffer concentrate - 21 ml of a 25-fold concentrated solution of buffered surfactant with preservatives.

Colour reagent A - 12.5 ml of stabilized hydrogen peroxide.

Colour reagent B - 12.5 ml of stabilized chromogen (tetramethylbenzidine).

Stop solution - 6 ml of 2 N sulphuric acid.

Plate covers - 4 adhesive strips.

Reagents supplied with TNFR-II kit:

sTNFR-II microplate - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against sTNFR-II.

sTNFR-II conjugate - 21 ml of polyclonal antibody against sTNFR-II conjugated to horseradish peroxidase, with preservatives.

sTNFR-II standard - 2.5 ng of recombinant human sTNFR-II in a buffered protein base with preservatives, lyophilized.

Assay diluent RD1-6 - 11 ml of a buffered protein base with preservatives.

Calibrator diluent RD5-5 - 2 vials (21 ml/vial) of a buffered protein base with preservatives.

Wash buffer concentrate - 21 ml of a 25-fold concentrated solution of buffered surfactant with preservatives.

Colour reagent A - 12.5 ml of stabilized hydrogen peroxide.

Colour reagent B - 12.5 ml of stabilized chromogen (tetramethylbenzidine).

Stop solution - 6 ml of 2 N sulphuric acid.

Plate covers - 4 adhesive strips.

Working reagents:

Wash buffer - 20 ml of wash buffer concentrate was diluted to 500 ml with distilled water to prepare wash buffer.

Substrate solution - Colour reagents A and B were mixed together in equal volumes within 15 minutes of use.

sTNFR-I and sTNFR-II standards - sTNFR-I or sTNFR-II were reconstituted with 5 ml of calibrator diluent RD5-5 to make a stock solution of 500 pg/ml.

Assay procedure:

50 µl of assay diluents HD1-7 for sTNFR-I and RD1-6 for sTNFR-II were added to each well of microplate coated with mouse monoclonal antibody against sTNFR-I and sTNFR-II, respectively. Thereafter, 200 µl of standards and each sample (culture supernatants) were added to the wells and incubated for 2 hours at room temperature, followed by washing three times with wash buffer. 200 µl of sTNFR-I conjugate or sTNFR-II conjugate were added to each well, incubated for 2 hours for TNFR-I or 1 hour for TNFR-II at room temperature and washed thrice. Thereafter, 200 µl of substrate solutions were added and incubated for 20 minutes at room temperature, followed by the addition of 50 µl of stop solution to each well to stop the reaction. Absorbance of each well was determined at 450 nm using a microplate reader.

Measurement of *M. tuberculosis* 85 complex protein by ELISA:

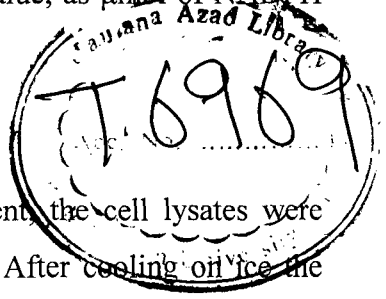
An ELISA for detection of the mycobacterial 85 complex (includes *M. tuberculosis* 85A, B, and C proteins) was used by the method of Islam et al. (2004). Immunoplates of 96-wells were coated with 100 µl of monoclonal antibody to mycobacterial 85 complex (CS-90; Colorado State University, Fort Collins) and incubated overnight at 4°C. The wells were then washed twice and then non-specific sites in the coated wells were blocked by the addition of 150 µl of 1.5% BSA and

incubated for 4–6 hours at room temperature. The blocking solution was then removed from the wells. 100 µl of the samples was added to the wells and incubated at 37°C for 90 minutes. The wells were again washed two times. 100 µl of polyclonal rabbit anti-*Mycobacterium bovis* antibody (Dako, Carpinteria, CA, USA) as detection reagent was added to the wells and incubated for 2 hours at room temperature. The wells were again washed four times. 100 µl of alkaline phosphatase-conjugated anti-rabbit IgG diluted 1:10,000 in TBS was added into the wells and incubated for 2 hours at room temperature. After another four washings, 100µl of p-NPP substrate solution was added into the wells and incubated at room temperature for about 30 minutes. The absorbance of each well was read at 405 nm on a microplate reader. Purified antigen 85 complex (Colorado State University) was used as a standard. The results from the specimens were compared to those of a serially diluted standard at 1–1000 pg/ml.

Glutathione peroxidase assay:

The activity of glutathione peroxidase (GPx) was measured as described elsewhere (Mohandas et al., 1984; Mates et al., 1999). The oxidized glutathione (GSSG) produced during GPx reaction was immediately reduced by NADPH and glutathione reductase. Therefore, the rate of NADPH consumption was regarded as the rate of GSSG formation during the GPx reaction. *M. tuberculosis*-infected monocytes were co-cultured for 24 hours with or without 10 mM NAC, 100 µg/ml SN50, 100 µg/ml SN50/M and 0–500 ng/ml allicin in the presence or absence of 10 nM H_2O_2. Thereafter, cells were gently scraped with lysis buffer containing protease inhibitors (50 mM Tris/HCl, pH 7.4; 1 mM EDTA; 500 mM PMSF). The cell suspension was homogenized with a sonicator on ice and centrifuged at 10,000 rpm for 10 minutes. Protein concentrations of supernatants were determined by the method of Bradford with BSA as the standard, and were subjected to GPx activity determination. The reaction mixture (1.0 ml) containing 50 mM potassium phosphate (pH 7.0), 1 mM sodium azide, 2 mM GSH, 0.2 mM NADPH, 1 unit/ml glutathione reductase, 1.5 mM cumene hydroperoxide, and 20–100 µl of samples were incubated at 25°C for 5 minutes. The reaction was initiated by the addition of cumene hydroperoxide. The kinetic change was spectrophotometrically recorded at 340 nm (37°C) for 3 minutes.

GPx activity was calculated after subtraction of the blank value, as μmol of NADPH oxidized/minute/mg protein (U/mg protein).



RNA extraction:

After lysis of monocytes in 0.5 ml TRIZOL Reagent, the cell lysates were agitated with glass beads to complete cell wall disruption. After cooling on ice the tubes were again subjected to repeated disruption as above. The tubes were cooled and 200 μl of chloroform was added to each sample, followed by vortexing for 2 minutes, and centrifugation at 3000 rpm for 5 minutes. Samples were then transferred to fresh eppendorf tubes and centrifuged at 14,000 rpm for 15 minutes at 4°C. The aqueous layer was harvested and transferred to a fresh tube. After addition of 100 μl Cleanascite (CPG Inc., Lincoln Park, NJ, USA), samples were gently rocked for 10 minutes and then centrifuged at 14,000 rpm for one minute. The aqueous layer obtained was mixed with 500 μl of chloroform-isoamyl alcohol (24:1) and vortexed. RNA was precipitated using 50 μl of 1 M sodium acetate, and 475 μl of isopropanol at –20°C for 3 hours in the presence of glycogen. This was followed by centrifugation at 14,000 rpm and the pellet obtained was washed two times with 75% ethanol, and resuspended in 87 μl DEPC-water. DNAase 1 digestion (10 μl of 10X DNAse 1 buffer in 0.5 M Tris pH 7.5, 0.1 M $MgCl_2$, 1 mM DTT; and 50 μg/ml BSA, 2.0 μl RNAase inhibitor; 10U RNAase free DNAase 1) was employed to remove DNA. The reaction was stopped by the addition of 700 μl of 0.5 M NH_4OAc and the RNA was re-extracted using 500 μl of acid phenol-chloroform (1:1). The aqueous layer was harvested, extracted again with chloroform-isoamyl alcohol and precipitated.

Reverse transcriptase polymerase chain reaction (RT-PCR):

The DNAase-treated RNA was subjected to reverse transcription using oligo(dT) primers with SuperScript II reverse transcriptase (Invitrogen, Life Technologies, USA) according to the manufacturer's instructions. RNA (2 μg) was transcribed into cDNA in a 20 μl reaction volume containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM of each deoxynucleotide triphosphate, 25 μg/ml oligo$(dT)_{12-18}$ primers and 10 U/μl of SuperScript II reverse

transcriptase, at 42°C for 50 minutes. The reaction was then stopped by heating at 70°C for 15 minutes followed by rapid chilling on ice.

PCR: The primers used in the PCR are listed in Table 1. cDNA for β-actin was amplified with various primer sets supplied by (Stratagene, La Jolla, CA, USA). For PCR, 2.0 µl of each cDNA sample was used as template in the PCR amplification. The reactions were carried out in a 50 µl reaction volume containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each of the four dNTPs, 2U of Taq DNA polymerase (Invitrogen) and 0.2 µM of each forward and reverse primers. After initial denaturation for 2 minutes at 95°C, 35 cycles at 95°C for 15 seconds, 60°C for 45 seconds were performed, followed by 72°C for 1 minute. The reaction products were visualized by electrophoresis in 2% agarose after staining with 0.5 µg/ml ethidium bromide.

Quantitative real-time RT-PCR:

Real-time quantitative reverse transcriptase PCR (RT-PCR), which is the latest innovation in the field of PCR technology, provides a sensitive, reproducible, and accurate method for determining mRNA levels in tissues or cells. The method is based on the detection of a fluorescent signal produced and monitored during the amplification process, without the need for post-PCR processing (Heid et al., 1996).

Two important findings led to the discovery of real-time PCR. First, the Taq polymerase has a 5′–3′ exonuclease activity (Holland et al., 1991), apart from its polymerase activity. Second, dual-labelled fluorogenic oligonucleotide probes have been created which emit a fluorescent signal only upon cleavage, based on the principle of fluorescence resonance energy transfer (Cardullo et al., 1988). In the TaqMan assay (Applied Biosystems, Foster City, CA, USA), these two principles are combined. In this system a probe, the so-called TaqMan probe, is designed to anneal to the target sequence between the classical forward and reverse primers. The probe is dually labelled, with a reporter fluorochrome (eg., 5-carbofluorescein, or FAM) at one end and a quencher dye (eg., N,N,N′,N′-tetramethyl-6-carborhodamine, or TAMRA) at the other end. In the intact probe, the fluorescence emission of the reporter dye will be absorbed by the quencher dye. The probe will be degraded during the extension

Table 1. Sequences of primers and probes for quantification of human TNF-α and *M. tuberculosis* 85B mRNA in infected monocytes.

Target, strain, primer	PCR primer sequence, 5′→3′	Taqman probe sequence, 5′→3′
Human		
TNF-α		CCAGAGGGAAGAGTTCCCCAGGGAC
RT	GGTTTCTACAACA	
Forward	AGGCGGTGCTTGTTCCTCA	
Reverse	GTTCGAGAAGATGATCTGACTGCC	
R18		ACCGGCGCAAGACGGACCAGA
RT	GACGGTATCTGATC	
Forward	CGCCGCTAGAGGTGAAATTC	
Reverse	CATTCTTGGCAAATGCTTTC	
M. tuberculosis		
85B		TCGAGTGACCCGGCATGGGAGCG
RT	TGTTGTTTGCGA	
Forward	TCAGGGGATGGGGCCTAGCC	
Reverse	GCTTGGGGATCTGCTGCGTA	
16S		AGCACCGGCCAACTACGTGCCAG
RT	CCCAGTAATTCC	
Forward	TTCTCTCGGATTGACGGTAGGT	
Reverse	CGCTCGCACCCTACGTATTAC	

phase by the 5′–3′ exonuclease activity of the Taq polymerase, separating the reporter and quencher, thus resulting in an increase in reporter fluorescence emission. The amount of fluorescence released is directly proportional to the amount of product generated in each PCR cycle and thus can be applied as a quantitative measure of PCR product formation.

Procedure:

Internal fluorescent hybridization probes were used in ABI Prism 7700 Detection System (ABI/PerkinElmer (PE) Biosystems, Foster City, CA, USA), which allows the sensitive and specific quantification of individual host (Hartel et al., 1999), as well as *M. tuberculosis* RNA, transcripts (Wilkinson et al., 2001) by quantitative real-time RT-PCR. TaqMan^{TM} PCR primers and probes as well as target-specific RT primer for each assay were designed as described elsewhere (Wilkinson et al., 2001; Islam et al., 2004). The primer and probe sequences used as depicted in Table 1 have been previously reported (Islam et al., 2004). The primer and probe combination for *M. tuberculosis* 85B is specific for 85B and does not detect the closely related 85A or 85C sequences. All probes were dually labeled with FAM at the 5′ end and TAMRA at the 3′ end. The proximity of the dye (FAM) and the quencher (TAMRA) on the intact probe prevents detection of any fluorescence. However, degradation of the probe during the course of PCR allows the release and detection of FAM (Holland et al., 1991). The PCRs for all amplifications were similar: 5 µl of each cDNA, 20 µl of Taqman Universal PCR Master Mix (PE Biosystems), which contains optimal amounts of AmpliTaq Gold DNA polymerase (which protects against amplicon carryover) and of dNTPs, and optimal amounts of probe and primers calibrated to allow measurement of the targets. First, cDNA was synthesized in the presence of 0.5 µl of murine leukemia virus enzyme (Invitrogen, USA)/reaction and 10 µM each RT primer, dNTPs, and other substrate. Conditions for PCR were similar for all products (1 cycle of 2 minutes at 50°C and 1 cycle of 10 minutes at 95°C and then 40 cycles of 15 seconds at 95°C and 1 minute at 60°C). The cycle threshold for each sample was compared with the cycle threshold values of known amounts of a standard DNA constructed for each target and amplified simultaneously. To assure lack of DNA contamination in the RNA samples, in some experiments, a duplicate tube of sample

with no RT enzyme was included as control. DNA contamination remained negligible. In each sample, host 18S ribosomal RNA was used as the internal control. Expression of TNF-α mRNA was corrected to internal control (host 18S rRNA) in the same sample and was expressed as copies of TNF-α in 10^{10} copies of R18 (equivalent to 1 x 10^6 monocytes). *M. tuberculosis* 85B mRNA was corrected to mycobacterial 16S rRNA in the same sample and expressed as 85B:16S.

Statistical analysis:

Results were analyzed by paired t-test and the data expressed as mean ± SEM of six experiments unless otherwise specified. $P<0.05$ was considered statistically significant.

Results

(A) Characterization of Sera and Monocytes from Tuberculosis Patients:

Direct binding and inhibition ELISA:

An attempt was made to probe the reactivity of sera from tuberculosis patients (n=15) used in this study, against *M. tuberculosis* antigens secreted in *M. tuberculosis* (H37Rv) culture filtrate (MTCF) as well as against intracellular protein antigens in bacilli sonic extract (MTSE). Direct binding ELISA on microtitre plates coated with total protein antigens of MTSE showed remarkable reactivity against antibodies found in sera of tuberculosis patients, as evident from Table 2, where most of the sera exhibited an antibody titre >1:12800. No binding was observed with normal human sera. Similarly, an appreciable reactivity was observed with tuberculosis sera against secreted MTCF protein antigens that were coated on microtitre plates. Here, most of the sera showed an antibody titre >1:6400 (Table 2). Thus, the results show that all the patients selected in this study had a high degree of active tuberculosis.

After detecting the reactivity of selected tuberculosis sera against *M. tuberculosis* antigens, we attempted to determine the specificity by employing inhibition ELISA. The data obtained from competition inhibition ELISA, depicted in Table 3, exhibited high magnitude inhibition of tuberculosis sera antibody activity with MTSE and MTCF protein antigens. A maximum of 76.4% inhibition in antibody activity was achieved at a maximum inhibitor concentration of 20 μg/ml of MTSE protein antigens. 50% inhibition was recorded at an inhibitor concentration of 0.7 μg/ml. Similarly, with MTCF proteins as an inhibitor, a maximum of 70.2% inhibition in the antibody activity was observed at a maximum inhibitor concentration of 20 μg/ml, where 50% inhibition was achieved at an inhibitor concentration of 0.9 μg/ml. Thus, inhibition data showed that the intracellular protein antigens, liberated from bacilli by sonication, as well as secretory MTCF protein antigens have high magnitude of specificity for tuberculosis antibody. Thereafter, tuberculosis IgG was isolated from serum by protein A sepharose CL-4B affinity chromatography. The purity of IgG was ascertained by SDS-PAGE under non-reducing conditions (Fig. 5), with the IgG appearing as a single band. This was employed in ELISA. The isolated IgG showed a very high degree of specificity towards MTSE as well as MTCF proteins, as only

Table 2. Direct binding ELISA of *Mycobacterium tuberculosis* sonic extract (MTSE) and culture filtrate (MTCF) protein antigens with tuberculosis antibodies.

Number of tuberculosis sera	MTSE protein antigens	MTCF protein antigens
	Antibody titre*	Antibody titre*
1	> 1:12800	≥ 1:6400
2	> 1:6400	≥ 1:3200
3	> 1:12800	> 1:3200
4	> 1:12800	≥ 1:6400
5	≥ 1:12800	> 1:6400
6	> 1:12800	≥ 1:6400
7	≥ 1:12800	> 1:6400
8	> 1:12800	> 1:6400
9	> 1:12800	> 1:6400
10	≥ 1:6400	≥ 1:3200
11	> 1:6400	≥ 1:6400
12	> 1:12800	> 1:6400
13	> 1:12800	> 1:6400
14	≥ 1:12800	> 1:6400
15	> 1:12800	> 1:6400

The microtitre plates were coated with MTSE and MTCF proteins (30 μg/ml).

* Antibody titres of tuberculosis sera were calculated with respect to normal human sera.

Table 3. Competition ELISA of *Mycobacterium tuberculosis* sonic extract (MTSE) and culture filtrate (MTCF) protein antigens with tuberculosis antibodies.

Inhibitor ►	**MTSE protein antigens**		**MTCF protein antigens**	
Tuberculosis sera ▼	Concentration for 50% inhibition (μg/ml)	Maximum inhibition (at 20 μg/ml) (%)	Concentration for 50% inhibition (μg/ml)	Maximum inhibition (at 20 μg/ml) (%)
1	0.6	72.8	0.9	70.4
2	1.0	75.1	0.5	71.6
3	0.8	76.4	1.3	69.5
4	0.1	79.2	0.2	72.5
5	1.1	71.6	1.2	68.6
6	0.6	76.9	1.8	66.6
7	0.5	77.8	0.9	69.7
8	0.6	78.9	0.7	71.8
9	1.5	70.5	1.6	68.5
10	1.3	72.7	1.3	68.7
11	1.1	75.5	0.8	70.6
12	0.3	80.3	1.2	67.9
13	0.1	82.2	0.3	73.6
14	0.5	78.5	0.4	71.5
15	0.4	77.6	0.3	72.5
Mean ± SD	**0.7 ± 0.4**	**76.4 ± 3.3**	**0.9 ± 0.5**	**70.2 ± 2.0**
TB IgG	0.08	89.0	0.09	75.0

The microtitre plates were coated with MTSE and MTCF proteins (30 μg/ml).

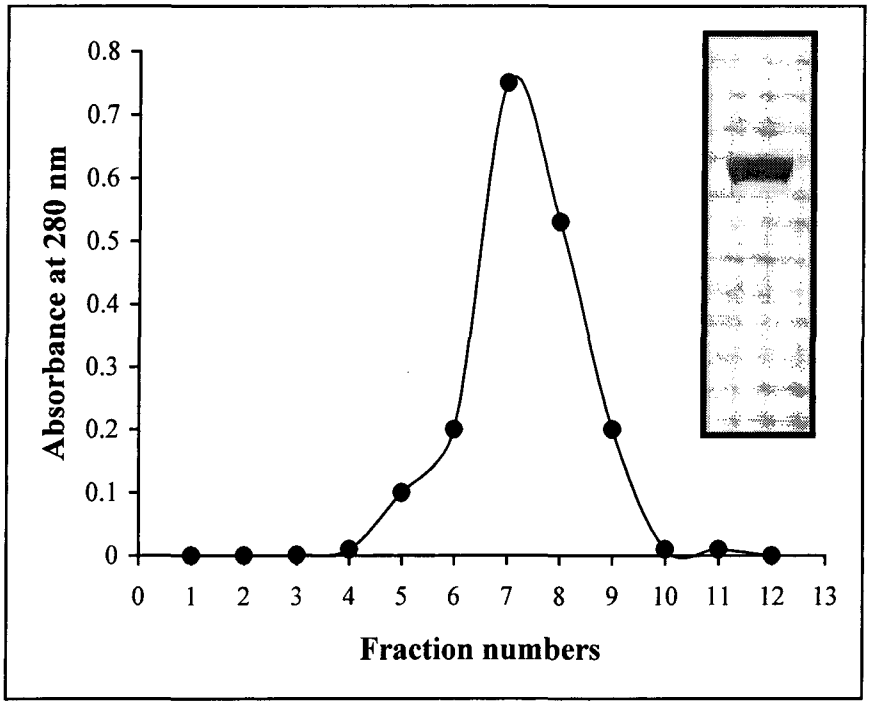


Figure 5. Elution profile of tuberculosis IgG with 0.58% acetic acid and 0.85% NaCl on protein A sepharose CL-4B column. Inset shows single band of IgG on 7.5% SDS-PAGE.

0.08 and 0.09 μg/ml, respectively, of the protein inhibitors were sufficient to cause 50% depletion in the TB-IgG activity (Table 3).

Quantification of soluble TNF-α in sera of tuberculosis patients by ELISA:

Since TNF-α is an autocrine cytokine known to have both deleterious as well as beneficial effects in tuberculosis, an attempt was made to detect and quantify the levels of soluble TNF-α present in sera of patients with active tuberculosis selected in this study, in comparison to sera from normal healthy individuals. ELISA results depicted in Fig. 6 clearly show an appreciable magnitude of soluble TNF-α present in sera of tuberculosis patients. As is evident from the data, a mean ± SEM concentration of soluble TNF-α was 207 ± 28 pg/ml ($P<0.001$) in sera from tuberculosis patients (n=15), whereas no or negligible sTNF-α was detected in sera from normal healthy individuals (n=15).

Quantification of circulating *M. tuberculosis* antigen 85 complex in sera of tuberculosis patients by ELISA:

The presence of circulating *M. tuberculosis* 85B antigen (30 kDa) in sera of tuberculosis patients, either in the free form or complexed with fibronectin has been well established. Thus, in the present study, the selected tuberculosis sera were further characterized by subjecting them to evaluation of *M. tuberculosis* antigen 85 complex. The mean ± SEM concentration of *M. tuberculosis* 85 complex detected in sera of tuberculosis patients was 685 ± 55 pg/ml (n=15) (Fig. 7), whereas *M. tuberculosis* 85 complex was not detected in sera from normal healthy individuals (n=15).

Real-time RT-PCR of TNF-α and *M. tuberculosis* 85B mRNA expression in monocytes of tuberculosis patients:

After characterizing sera from tuberculosis patients, we attempted the characterization of monocytes from tuberculosis patients, with respect to TNF-α and 85B. PBMCs were isolated from the blood of tuberculosis patients to obtain adherent monocytes as described in the experimental section. Monocytes from tuberculosis patients were subjected to TNF-α mRNA evaluation by real-time RT-PCR. Monocytes from healthy individuals served as control. As evident from Fig. 8, in comparison to control monocytes, the monocytes of tuberculosis patients revealed the presence of

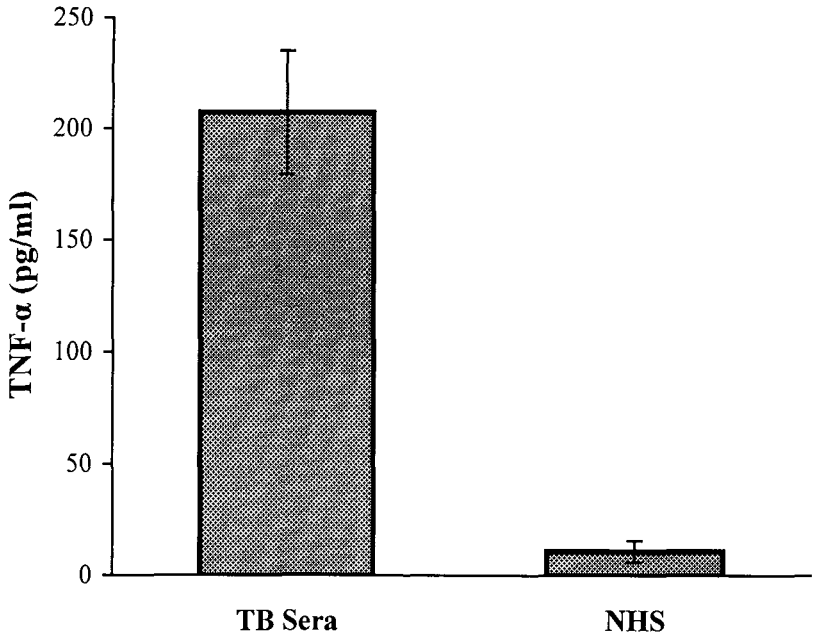


Figure 6. ELISA for TNF-α: Soluble TNF-α was determined in sera of tuberculosis patients and normal healthy individuals. Data represent mean ± SEM of 15 patients.

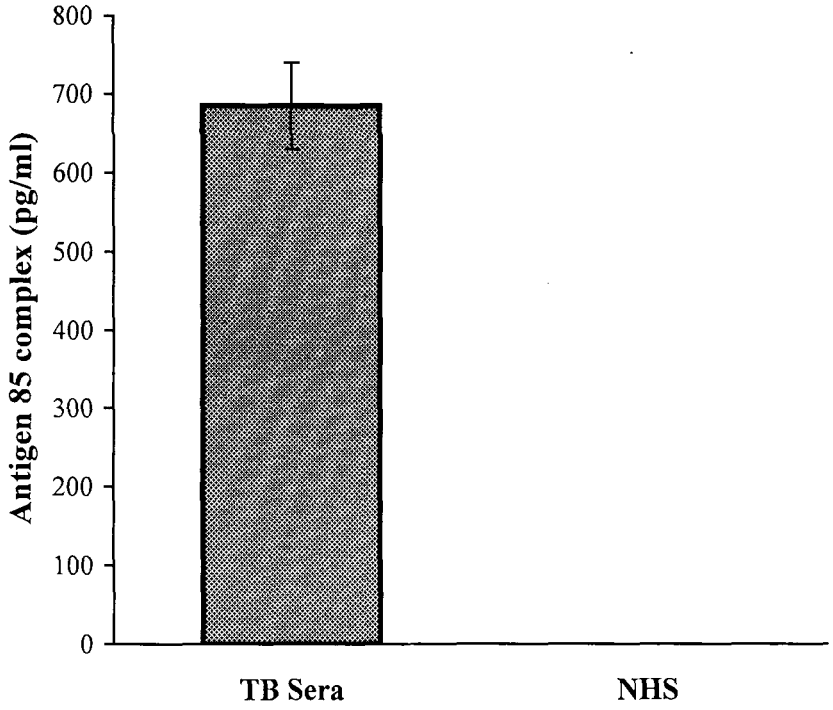


Figure 7. ELISA for antigen 85 complex: Secreted antigen 85 complex was determined in sera of tuberculosis patients and normal healthy individuals. Data represent mean ± SEM of 15 patients.

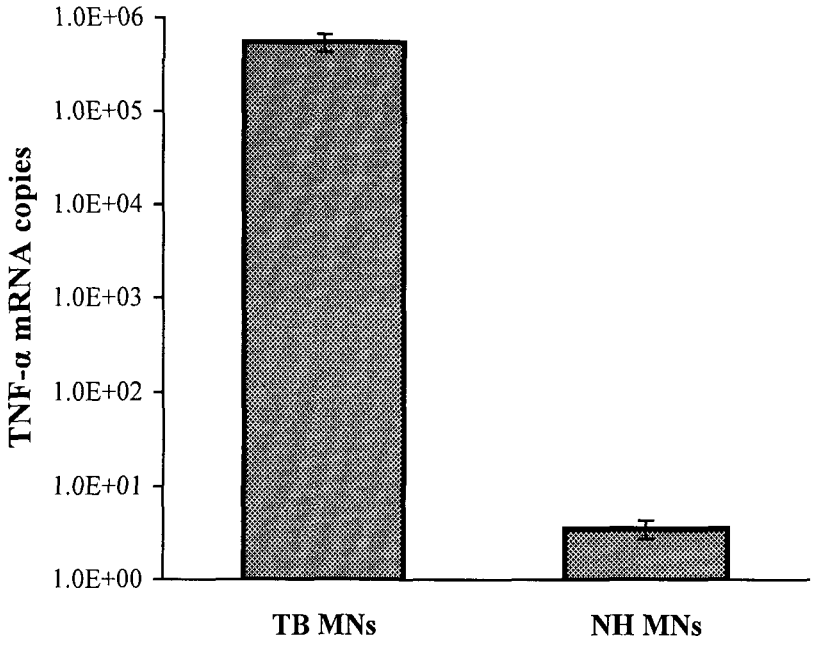


Figure 8. Real-time RT-PCR for TNF-α mRNA expression: Monocytes of tuberculosis patients and normal healthy individuals were cultured in RPMI-1640 medium. Total RNA was extracted and assessed for TNF-α mRNA. Data represent mean ± SEM of 4 experiments.

high basal levels of TNF-α mRNA copy number which was to the order of 5.47E+05 (P<0.001), i.e., basal levels of ~5 logs. Similarly, evaluation for *M. tuberculosis* 85B mRNA in the same monocytes by real-time RT-PCR showed appreciably high basal levels of 85B mRNA expression (~4.5 logs) in monocytes of tuberculosis patients as compared with control cells (Fig. 9). Thus, the data depicted in figures 8 and 9 revealed appreciably high basal levels of TNF-α and 85B mRNA in monocytes of patients with active tuberculosis.

Detection of soluble TNF-α and *M. tuberculosis* antigen 85 complex in supernatants of monocyte cultures of tuberculosis patients:

Adherent monocytes from PBMCs of tuberculosis patients were cultured for 24 hours and the supernatants obtained were subjected to ELISA for evaluation of soluble TNF-α as well as secreted *M. tuberculosis* 85 complex. 24 hours culture supernatants of tuberculosis monocytes exhibited soluble TNF-α to be present in the order of 235 ± 33 pg/ml (mean ± SEM; P<0.001) (Fig. 10), whereas, negligible and insignificant amounts of soluble TNF-α were observed in supernatants of normal healthy monocyte cultures.

The concentration of antigen 85 complex in 24 hours culture supernatants of tuberculosis monocytes was found to be 350 ± 41 pg/ml (mean ± SEM; P<0.001) (Fig. 11). Negligible amounts of antigen 85 complex were observed in supernatants of normal healthy monocyte cultures. As noted elsewhere (Wallis et al., 1998), *M. tuberculosis* 85B is a component of the *M. tuberculosis* antigen 85 complex, and changes in the antigen 85 complex immunoreactivity in supernatants of monocyte cultures or in sera likely reflect changes in 85B.

(B) Preliminary Studies Probing the Identification of a Natural Antagonist of *M. tuberculosis* 85B:

After ensuring that our laboratory protocols yield established results with minor variation, and prior to any further in-depth studies, we undertook preliminary studies to identify a natural antagonist of *M. tuberculosis* 85B. The objective of these experiments was to explore an economical and cost-friendly option towards combating tuberculosis. Taking into consideration the role of garlic in alternative medicine since ancient times, we opted to study its active component, allicin.

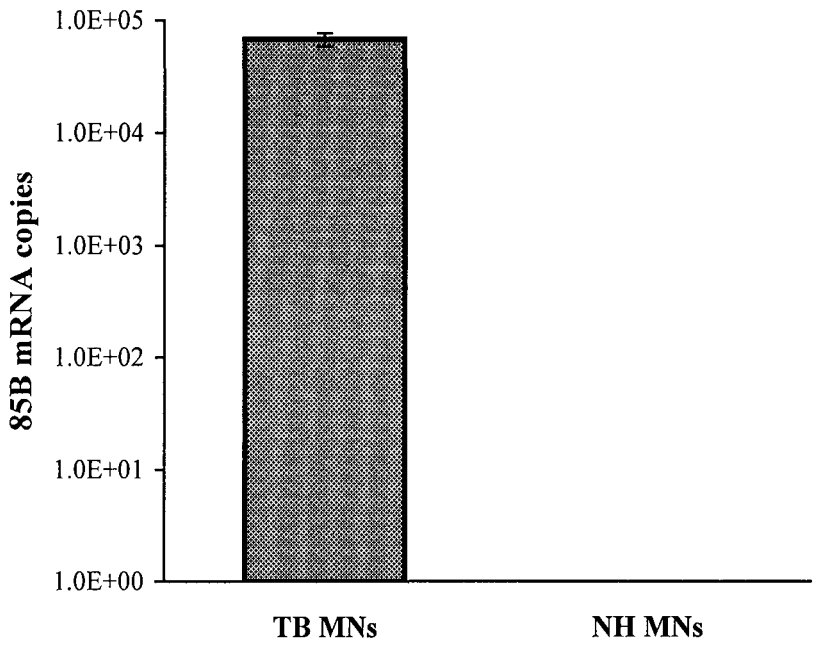


Figure 9. Real-time RT-PCR for *M. tuberculosis* 85B mRNA expression: Monocytes of tuberculosis patients and normal healthy individuals were cultured in RPMI-1640 medium. Total RNA was extracted and assessed for *M. tuberculosis* 85B mRNA. Data represent mean ± SEM of 4 experiments.

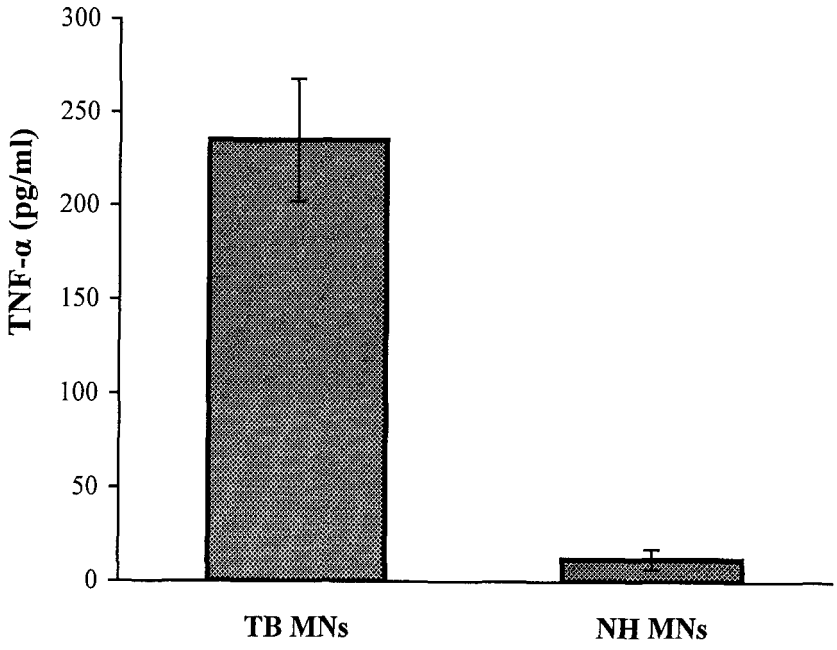


Figure 10. ELISA for TNF-α: Soluble TNF-α was determined in supernatants of monocyte cultures of tuberculosis patients and normal healthy individuals. Data represent mean ± SEM of 10 patients.

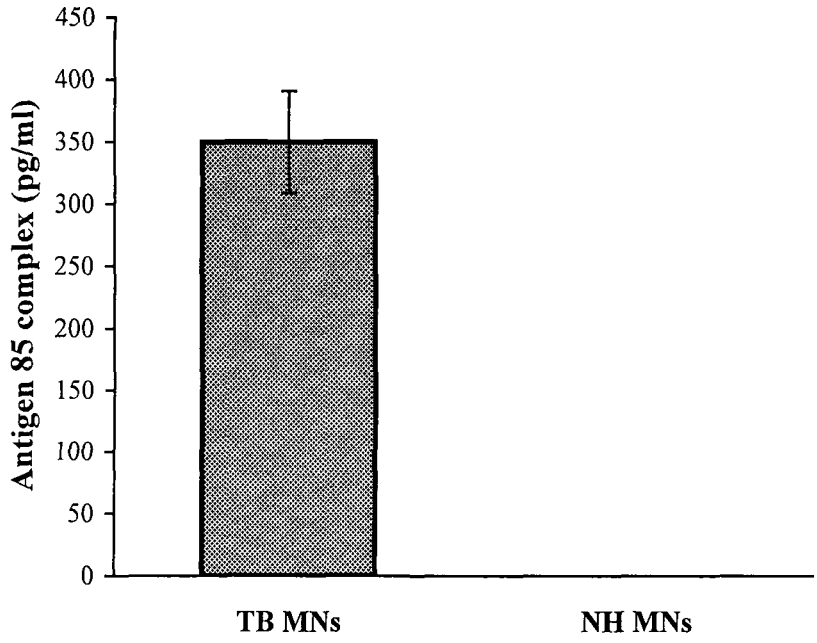


Figure 11. ELISA for antigen 85 complex: Secreted antigen 85 complex was determined in supernatants of monocyte cultures of tuberculosis patients and normal healthy individuals. Data represent mean ± SEM of 10 patients.

Dose-response effect of allicin on *M. tuberculosis* 85B mRNA expression in H37Rv cultures:

M. tuberculosis (H37Rv) co-cultured with various concentrations of allicin (50, 100, 250 and 500 ng/ml), as well as culture devoid of allicin which served as the corresponding control, were grown in Middlebrook 7H9 broth for 14 days at 37°C in 5% CO_2. After lysis of bacilli, RNA was extracted and assessed for 16S rRNA and 85B mRNA. None of the concentrations of allicin had any significant effect on the expression of the housekeeping gene of *M. tuberculosis*, i.e., 16S rRNA (Fig. 12), thereby indicating that allicin did not non-specifically affect *M. tuberculosis* transcription. In contrast, the data showed appreciable effects of allicin on the expression of *M. tuberculosis* 85B mRNA. As is evident from Fig. 13, cultures of H37Rv, receiving 50 ng/ml allicin exhibited negligible changes in 85B mRNA level in comparison to control cultures devoid of allicin. A low magnitude decrease to the order of 0.5 logs ($P<0.05$) in *M. tuberculosis* 85B mRNA expression was recorded with 100 ng/ml allicin. Further increase in allicin concentrations to 250 and 500 ng/ml in H37Rv cultures exhibited an appreciable suppression in *M. tuberculosis* 85B mRNA by ~2 and 2.5 logs ($P<0.001$ for both), respectively, in comparison to control cultures (Fig. 13). The decrease in the 85B:16S ratio (Fig. 14) to the extent of 98% and 99% observed for cultures challenged with 250 and 500 ng/ml of allicin, respectively, in comparison to control cultures devoid of allicin, supports the above data. It is to be pointed out here that previous reports have shown *M. tuberculosis* growth to be affected by allicin at relatively much higher concentrations and not at the lower concentrations that have been employed in this study. Although low concentrations of allicin do not affect the *M. tuberculosis* growth, it appreciably downregulates the *M. tuberculosis* 85B gene expression.

Effect of allicin on the expression of antigen 85 complex in H37Rv cultures:

M. tuberculosis (H37Rv) was cultured in Middlebrook 7H9 broth for 14 days with varying concentrations of allicin (0, 50, 100, 250 and 500 ng/ml). Thereafter, cultures were harvested and the bacilli were subjected to sonication to liberate intracellular proteins. Protein content was estimated in the untreated/treated sonic extracts as well as culture supernatants (MTCF). It is to be pointed out here that prior

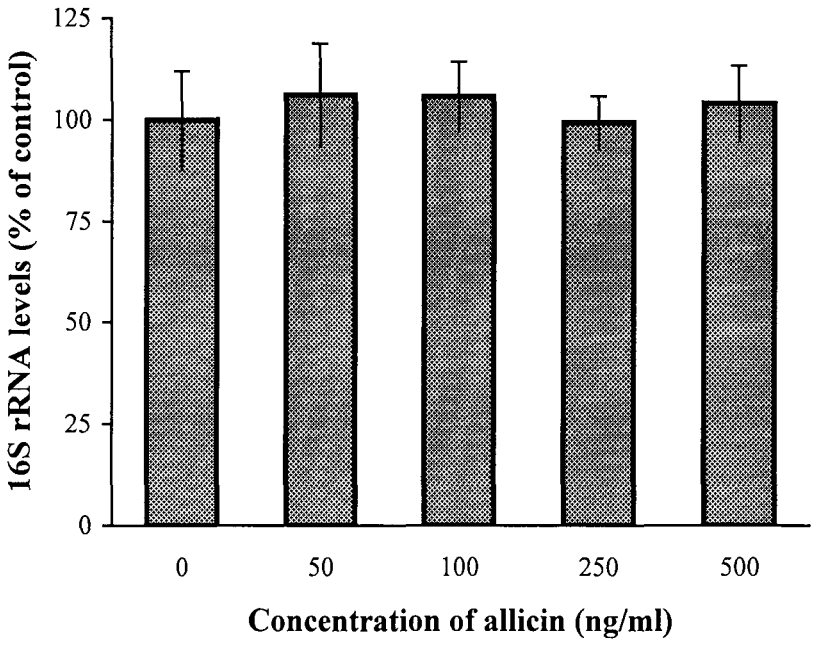


Figure 12. Real-time RT-PCR for expression of mycobacterial 16S rRNA: *M. tuberculosis* (H37Rv) was grown in Middlebrook 7H9 broth with different doses (0–500 ng/ml) of allicin. Midlogarithmic mycobacterial cultures (14 days) were harvested and total RNA was extracted and assessed for mycobacterial 16S rRNA. Data represent mean ± SEM of 4 experiments and expressed as percent of control cultures.

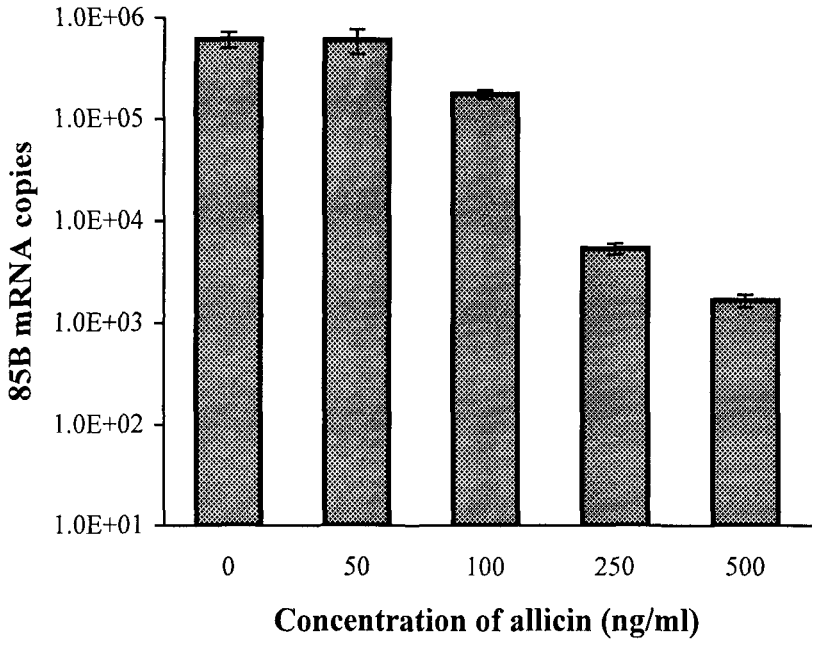


Figure 13. Real-time RT-PCR for *M. tuberculosis* 85B mRNA expression: *M. tuberculosis* (H37Rv) was grown in Middlebrook 7H9 broth with different doses (0–500 ng/ml) of allicin. Midlogarithmic mycobacterial cultures (14 days) were harvested and total RNA was extracted and assessed for *M. tuberculosis* 85B mRNA. Data represent mean ± SEM of 4 experiments.

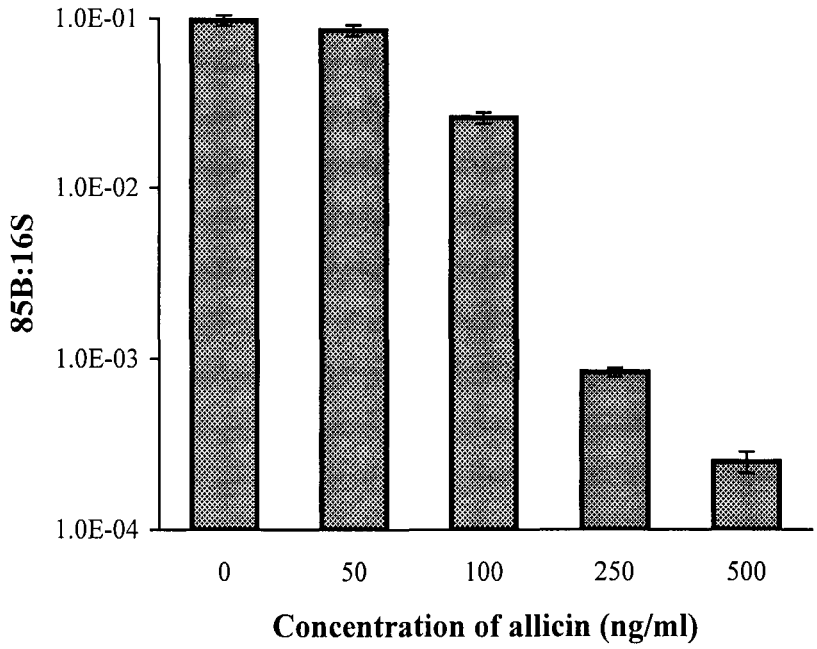


Figure 14. Real-time RT-PCR for expression of *M. tuberculosis* 85B:16S ratio: *M. tuberculosis* (H37Rv) was grown in Middlebrook 7H9 broth with different doses (0–500 ng/ml) of allicin. Midlogarithmic mycobacterial cultures (14 days) were harvested and total RNA was extracted and assessed for *M. tuberculosis* 85B:16S ratio. Data represent mean ± SEM of 4 experiments.

to any further study, the presence of 30/31 kDa protein band in the electrophoresed gel was ascertained by ELISA using anti-antigen 85 complex antibody. As evident from Fig. 15, the effect of allicin on expression of *M. tuberculosis* antigen 85 complex (30/31 kDa) in *M. tuberculosis* sonic extract as revealed by gradient SDS-PAGE was found to be dose-dependent. Thus, in comparison to control cultures devoid of any allicin (Lane 6), 250 and 500 ng/ml of allicin proved to be a potent inhibitor of antigen 85 complex (30/31 kDa) protein expression (Lanes 3 and 2, respectively).

In addition, supernatants obtained from 14 days *M. tuberculosis* cultures, treated with various doses of allicin were subjected to ELISA to evaluate secreted *M. tuberculosis* antigen 85 complex. As evident from Fig. 16, the secretion of antigen 85 complex was found to be dose-dependent and decreased by an appreciable amount with 250 and 500 ng/ml allicin. Hence, this decrease in antigen 85 complex secretion substantiates the above SDS-PAGE results.

Immunoassays for MTSE and MTCF protein antigens of *M. tuberculosis* (H37Rv) co-cultured with allicin, against TB-IgG:

Inhibition ELISA was performed to determine the tuberculosis antibody specificity on ELISA plates that were coated with sonic extract protein antigens of *M. tuberculosis* cultures. As evident from Fig. 17A, MTSE proteins obtained from cultures devoid of allicin exhibited a maximum of 89% inhibition in TB-IgG activity. 50% inhibition was observed at a very low inhibitor concentration (0.08 μg/ml). On the contrary, MTSE proteins of cultures receiving 50, 250, and 500 ng/ml allicin showed a maximum of 63%, 55%, and 49% inhibition in TB-IgG activity, respectively. MTSE proteins from cultures having 50 and 250 ng/ml allicin showed 50% inhibition at 7.0 and 13.0 μg/ml inhibitor concentrations, respectively. However, MTSE proteins obtained from cultures having 500 ng/ml allicin failed to show 50% inhibition.

Similarly, inhibition ELISA was performed to determine TB-IgG specificity using ELISA plates which were coated with MTCF protein antigens. As evident from Fig. 17B, MTCF proteins obtained from cultures devoid of allicin exhibited a

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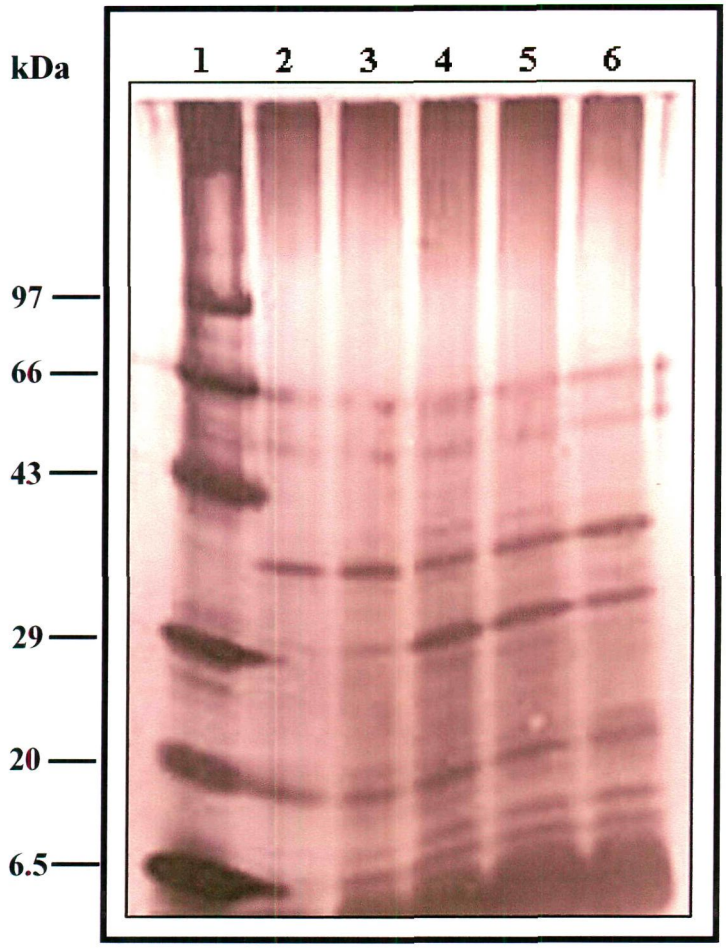


Figure 15. **SDS-PAGE of mycobacterial proteins: *Mycobacterium tuberculosis* (H37Rv) was grown in Middlebrook 7H9 broth with different doses (0–500 ng/ml) of allicin. Midlogarithmic mycobacterial cultures (14 days) were harvested and intracellular proteins were liberated by sonication and 100 µg of each sample was resolved by 10–20% gradient SDS-PAGE under reducing conditions and the gel was visualized by silver staining. Lane (1) molecular weight markers; Lanes 2–6 having 500, 250, 100, 50 and 0 ng/ml allicin, respectively.**

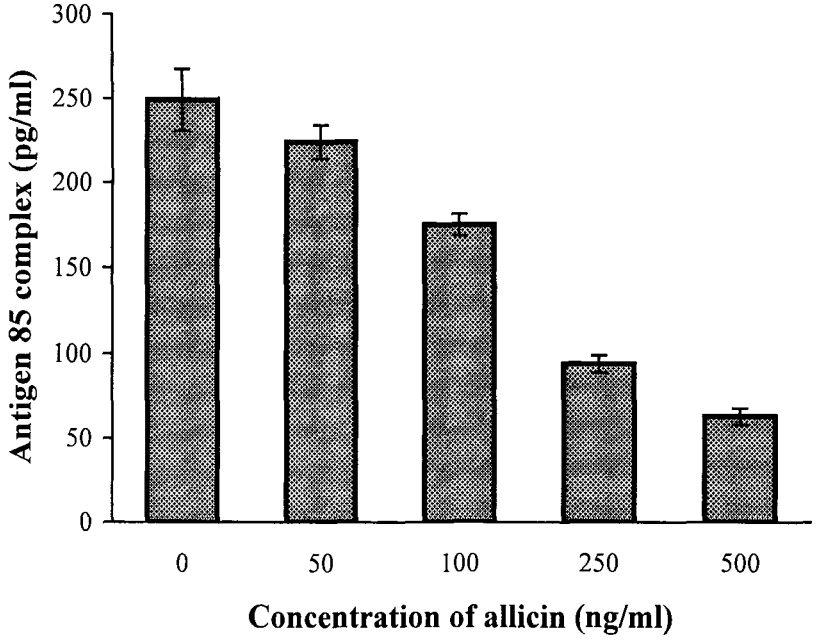


Figure 16. ELISA for antigen 85 complex: *M. tuberculosis* (H37Rv) was grown in Middlebrook 7H9 broth with different doses (0–500 ng/ml) of allicin. Midlogarithmic mycobacterial cultures (14 days) were harvested and secreted antigen 85 complex was determined in supernatants of cultures. Data represent mean ± SEM of 4 experiments.

THESIS

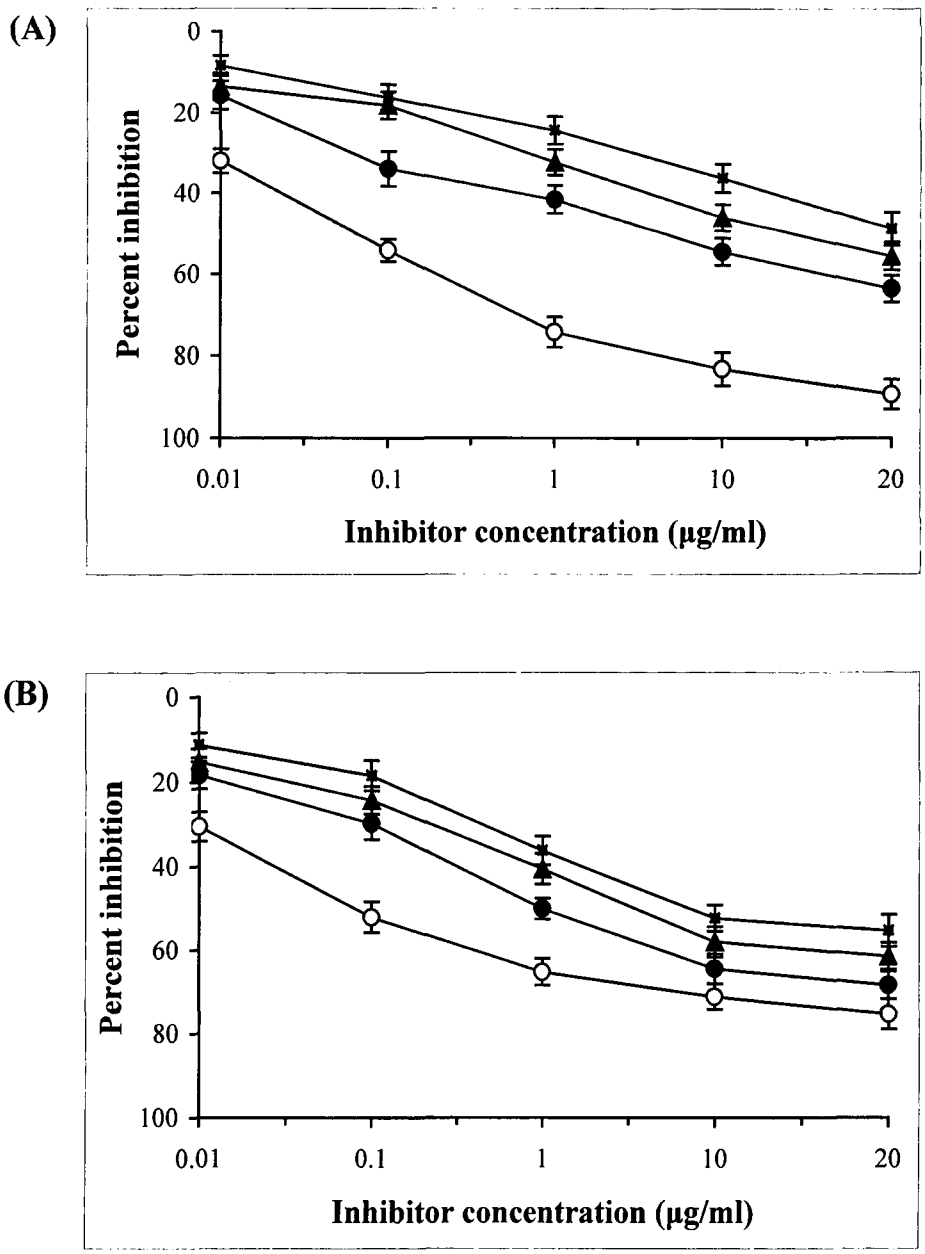


Figure 17. Inhibition ELISA of TB-IgG with mycobacterial proteins: *M. tuberculosis* (H37Rv) was grown in Middlebrook 7H9 broth with different doses (0–500 ng/ml) of allicin. Midlogarithmic mycobacterial cultures (14 days) were harvested and MTSE proteins as well as MTCF proteins were isolated. Inhibitors used were MTSE proteins (A) and MTCF proteins (B) having 0 ng/ml allicin (-o-), 50 ng/ml allicin (-•-), 250 ng/ml allicin (-▲-) and 500 ng/ml allicin (-■-). Microtitre plates were coated with (A) MTSE proteins (30 µg/ml) and (B) MTCF proteins (30 µg/ml). Each data represents mean ± SD from three duplicate experiments.

maximum of 75% inhibition in TB-IgG activity at an inhibitor concentration of 20 μg/ml. It is noteworthy that even at the onset of reaction, appreciable binding was seen. The high degree of specificity was evident from 50% inhibition achieved at an extremely low inhibitor concentration. Here, 50% inhibition in the activity of TB-IgG was achieved at an inhibitor concentration of 0.09 μg/ml. On the other hand, the degree of specificity of TB-IgG decreased tremendously in a somewhat linear manner when *M. tuberculosis* was co-cultured with varying doses of allicin. At a maximum inhibitor concentration of 20 μg/ml, a maximum of 68%, 61% and 55% inhibition in TB-IgG activity was achieved with MTCF proteins of cultures receiving 50, 250 and 500 ng/ml of allicin, respectively. 50% inhibition in TB-IgG activity was seen at appreciably higher inhibitor concentrations, i.e., 1.0, 6.0 and 9.0 μg/ml inhibitors, for 50, 250 and 500 ng/ml allicin-treated MTCF proteins, respectively (Fig. 17B). It is to be pointed out that the lower the amount of inhibitor required to achieve 50% inhibition in antibody activity, the more specific the immuno-interaction is. Thus, the results indicate that allicin decreases the specificity of TB-IgG for *M. tuberculosis* protein antigens.

Gel retardation assay:

The decrease in the specificity of IgG isolated from sera of patients with active tuberculosis against protein antigens of allicin-treated *M. tuberculosis* sonic extract and culture filtrate was further probed by gel retardation assay (Fig. 18). In comparison to control (Lane 1; 150 kDa IgG), a retarded mobility was observed for the mixture of IgG and protein antigens in total sonic extract (MTSE) (Lane 2, ~180 kDa), thereby indicating the formation of immune complex between IgG (150 kDa) and 30/31 kDa antigen 85 complex (Fig. 18A). To ascertain this, the above 180 kDa bands were spliced off and were used as coating antigen in ELISA against anti-antigen 85 complex antibodies, where a binding of high magnitude was observed. On the contrary, MTSE prepared from 500 ng/ml allicin-treated H37Rv cultures failed to show any binding with IgG as is evident from the band observed in lane 3. Similar observations were recorded with MTCF proteins as is evident from Fig. 18B.

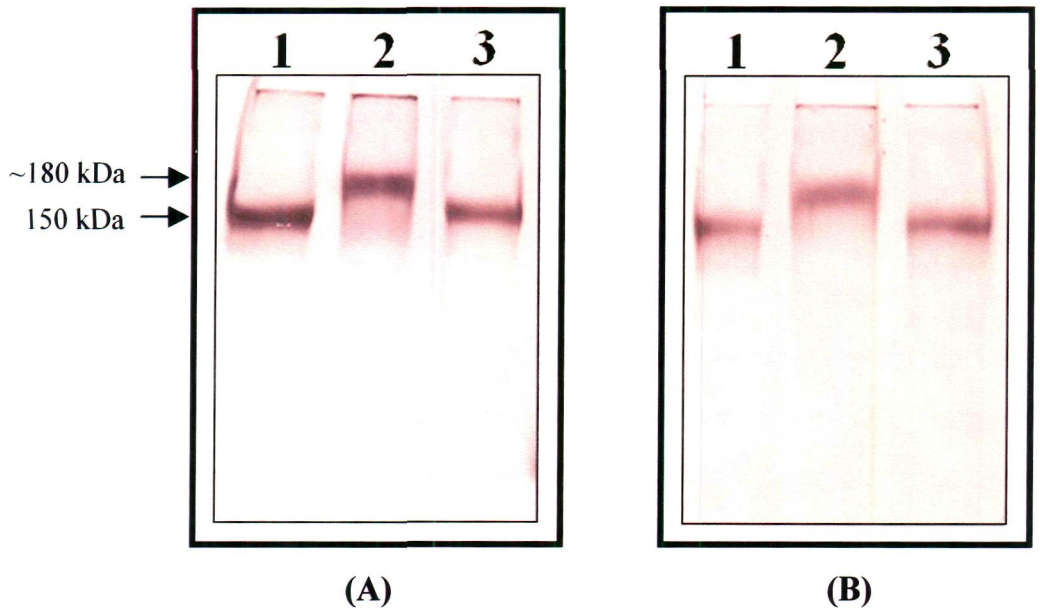


Figure 18. **Gel retardation assay: Detection of retarded mobility of immune complex formed between TB-IgG and antigen 85 complex in MTSE and MTCF on 7% SDS-PAGE. The resolved samples for assessing retarded band shift were; (A): TB-IgG as control (Lane 1; 150 kDa), immune complex of TB-IgG and antigen 85 complex in MTSE (Lane 2; ~180 kDa), immune complex of TB-IgG and 500 ng/ml allicin-treated MTSE (Lane 3); (B): TB-IgG as control (Lane 1; 150 kDa), immune complex of TB-IgG and antigen 85 complex in MTCF (Lane 2; ~180 kDa), immune complex of TB-IgG and 500 ng/ml allicin-treated MTCF (Lane 3).**

(C) Studies on Healthy Monocytes Infected *In Vitro* with *M. tuberculosis*:

Time-dependent effect of *M. tuberculosis* infection on the expression of human housekeeping genes and on the expression of TNF-α mRNA and secreted TNF-α:

An attempt was made to probe the effect of *M. tuberculosis* (H37Rv) infection, if any, on the human housekeeping genes. It was observed that *M. tuberculosis* infection had no effect at any time intervals (0–120 hours) of culturing of infected monocytes on the host housekeeping genes like R18 gene (18S rRNA) as revealed by quantitative real-time RT-PCR (Fig. 19) or β-actin gene as revealed by RT-PCR (Fig. 20).

Thereafter, we investigated the kinetics (0–120 hours) of expression of TNF-α mRNA in *M. tuberculosis*-infected monocytes. TNF-α mRNA was corrected to host internal control, i.e., 18S rRNA, in the same sample. As evident from Fig. 21A, the effect of *M. tuberculosis* infection on TNF-α mRNA was found to be time-dependent. The levels of TNF-α mRNA copy numbers in infected monocytes at various time intervals were recorded as 2.2 logs, 6.6 logs ($P<0.001$), 11.5 logs ($P<0.001$), 5.3 logs ($P<0.001$) and 2.1 logs ($P<0.05$), after 0, 4, 24, 72 and 120 hours of *M. tuberculosis* infection, respectively. Thus, the expression of TNF-α was maximal at 24 hours, which decreased considerably thereafter. A similar pattern of TNF-α mRNA expression was observed in RT-PCR results (Fig. 21B).

After this, we further probed the effect of *M. tuberculosis* infection on secreted TNF-α in culture supernatants that were harvested at various time intervals (0–120 hours). As evident from Fig. 22, the effect of *M. tuberculosis* infection on secreted TNF-α was found to be time-dependent. The concentration of soluble TNF-α secreted in supernatants of infected monocyte cultures at various time intervals was observed as 30.2, 65.0 ($P<0.001$), 93.2 ($P<0.001$), 194.3 ($P<0.001$), 112.2 ($P<0.001$), 65.8 ($P<0.001$) and 13.8 ($P<0.001$) pg/ml after 0, 4, 12, 24, 48, 72 and 120 hours of *M. tuberculosis* infection, respectively. Thus, the maximum secretion of TNF-α in culture supernatants was observed after 24 hours of *M. tuberculosis* infection, followed by a substantial decrease at the remaining time intervals of culture.

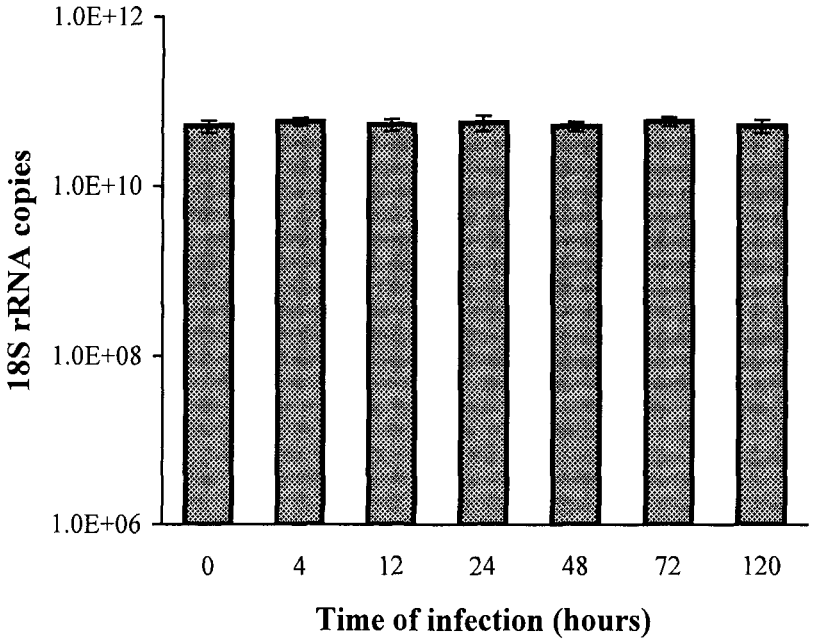


Figure 19. Real-time RT-PCR for kinetics of expression of host housekeeping gene: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured for 0–120 hours *in vitro*. Total RNA was extracted and assessed for host R18 gene (18S rRNA). Data represent mean ± SEM of 6 experiments.

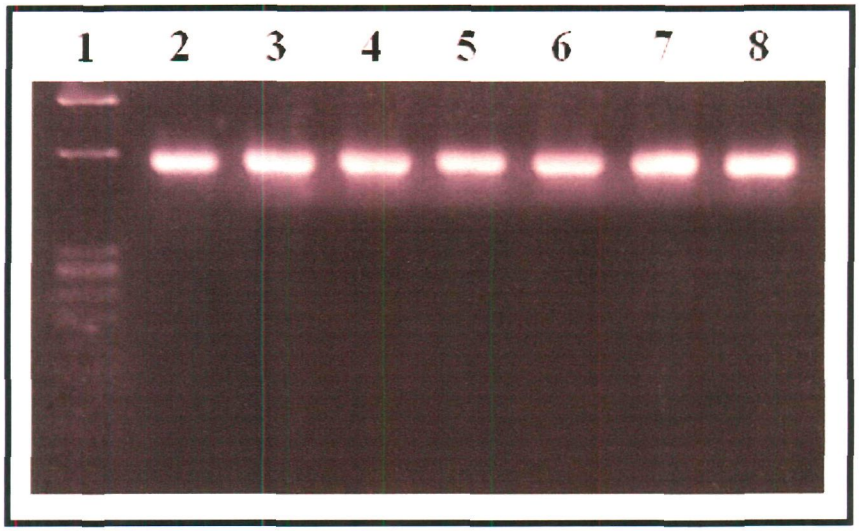


Figure 20. **RT-PCR for kinetics of expression of β-actin gene: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured for 0–120 hours *in vitro*. Total RNA was extracted and assessed for human housekeeping gene β-actin mRNA. The amplification product for β-actin (514 bp) is shown. Lane (1) DNA ladder; Lanes 2–8 for 0, 4, 12, 24, 48, 72, 120 hours of infection, respectively.**

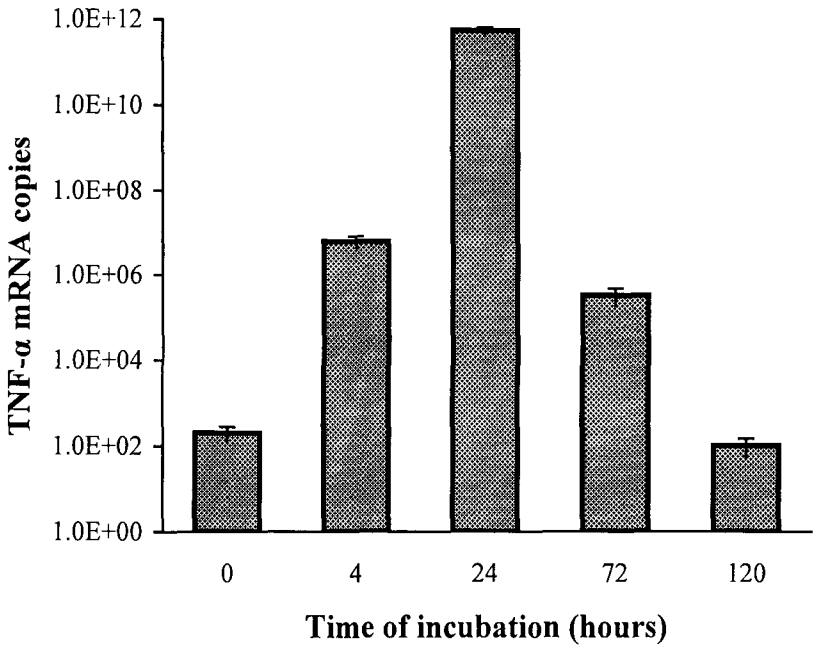


Figure 21A. Real-time RT-PCR for kinetics of TNF-α mRNA expression: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured for 0–120 hours *in vitro*. Total RNA was extracted and assessed for TNF-α mRNA. Expression of TNF-α was corrected to host 18S rRNA and expressed as copies of TNF-α in 10^{10} copies of R18 (equivalent to 1 x 10^6 monocytes). Data represent mean ± SEM of 6 experiments.

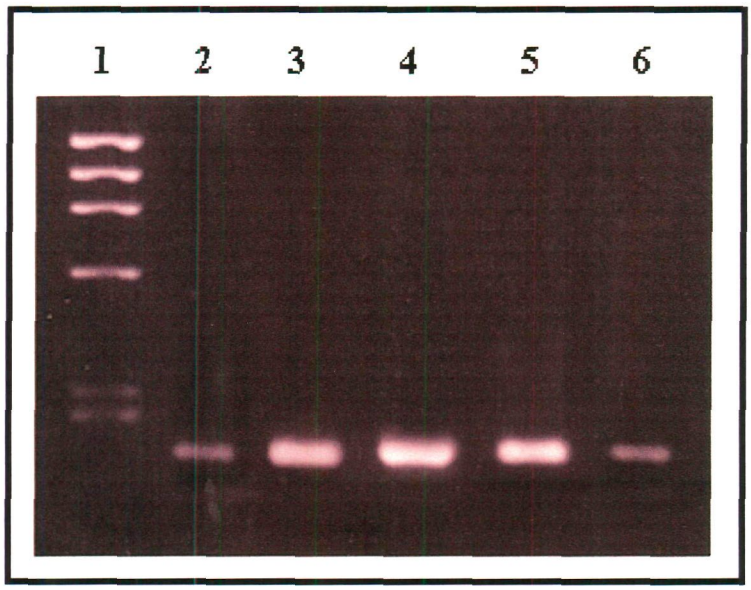


Figure 21B. **RT-PCR for kinetics of TNF-α mRNA expression: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured for 0–120 hours *in vitro*. Total RNA was extracted and assessed for TNF-α mRNA. The amplification product for TNF-α (342 bp) is shown. Lane (1) DNA ladder; Lanes 2–6 for 0, 4, 24, 72, 120 hours of infection, respectively.**

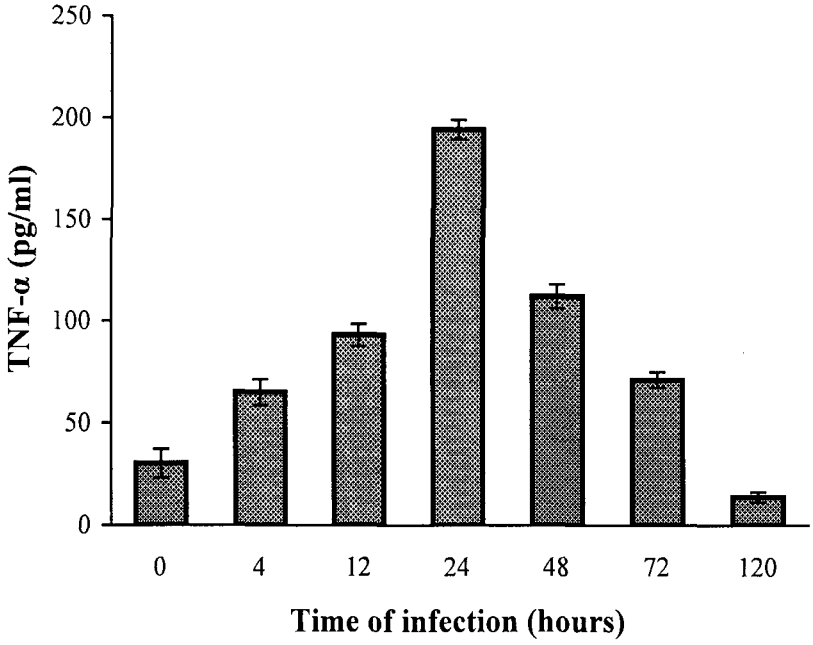


Figure 22. ELISA for kinetics of soluble TNF-α expression: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured for 0–120 hours *in vitro*. Soluble TNF-α was determined in supernatants of monocyte cultures. Data represent mean ± SEM of 6 experiments.

Time-dependent effect of *M. tuberculosis* (H37Rv) infection in healthy monocytes on *M. tuberculosis* 85B gene and on secreted antigen 85 complex:

We also investigated the kinetics (0–120 hours) of expression of *M. tuberculosis* 16S rRNA and 85B mRNA in *M. tuberculosis*-infected monocytes. As evident from Fig. 23, 16S rRNA expression was observed to linearly increase with time. Also, the expression of 85B mRNA continued to increase up to 120 hours (Fig. 24). The ratio of 85B:16S was found to increase with increasing time periods of infection. The 85B:16S ratio recorded at various time intervals were 0.0011, 0.0019 ($P<0.05$), 0.0875 ($P<0.001$), 0.3488 ($P<0.001$) and 0.7947 ($P<0.001$), after 0, 4, 24, 72 and 120 hours of infection, respectively (Fig. 25). Furthermore, between 4 and 24 hours, *M. tuberculosis* 85B:16S increased significantly ($P<0.001$). This increase in 85B:16S ratio with time correlates with 85B mRNA levels at various time periods observed in Fig. 24. Thereafter, we probed the kinetics (0–120 hours) of expression of secreted *M. tuberculosis* antigen 85 complex in the supernatants of *M. tuberculosis*-infected monocyte cultures. The expression of secreted antigen 85 complex was increased with increasing time intervals of infection (Fig. 26).

Time-dependent effect of *M. tuberculosis* infection of monocytes on glutathione peroxidase activity:

Time-course kinetics of GPx activity was determined in *M. tuberculosis*-infected monocytes. Uninfected monocytes served as controls. At zero time, GPx activity in uninfected control and *M. tuberculosis*-infected monocytes was observed to be nearly the same. No significant change in GPx activity was observed at any of the time intervals of culturing (0–120 hours) of uninfected control monocytes. However, in comparison to control monocytes, the *M. tuberculosis*-infected monocytes exhibited a linear decrease in GPx activity with time. GPx activity in *M. tuberculosis*-infected monocytes was recorded as 67.1, 55.3 ($P<0.02$), 40.1 ($P<0.01$), 34.7 ($P<0.001$) and 26.8 ($P<0.001$) U/mg protein, after 0, 4, 24, 72 and 120 hours of infection, respectively (Fig. 27).

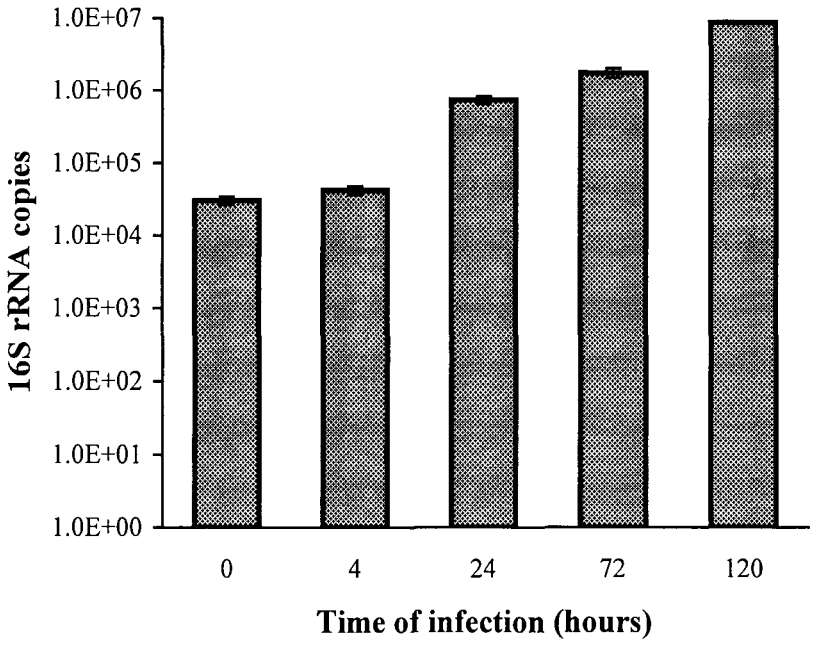


Figure 23. Real-time RT-PCR for kinetics of expression of mycobacterial housekeeping gene: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured for 0–120 hours in *vitro*. Total RNA was extracted and assessed for mycobacterial 16S rRNA. Data represent mean ± SEM of 6 experiments.

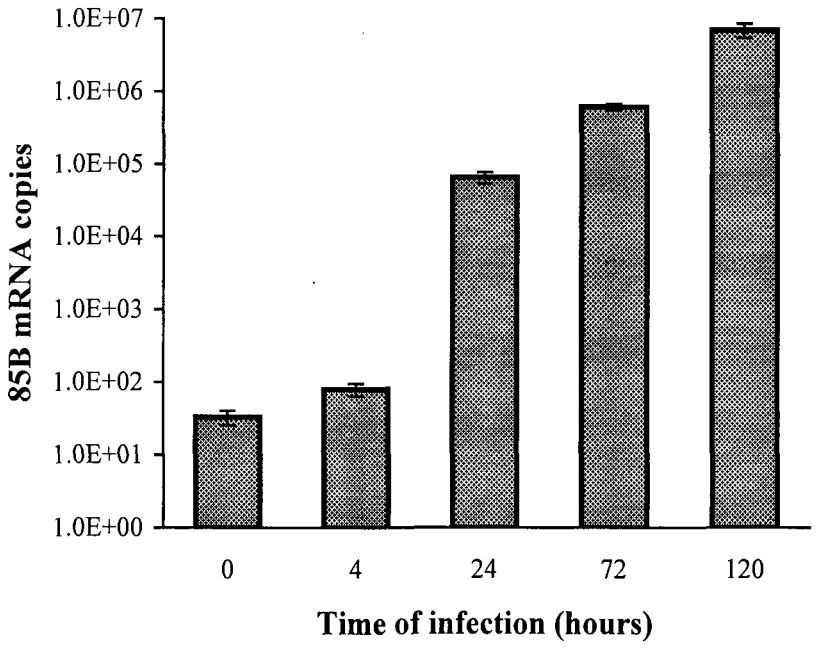


Figure 24. Real-time RT-PCR for kinetics of *M. tuberculosis* 85B mRNA expression: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured for 0–120 hours *in vitro*. Total RNA was extracted and assessed for *M. tuberculosis* 85B mRNA. Data represent mean ± SEM of 6 experiments.

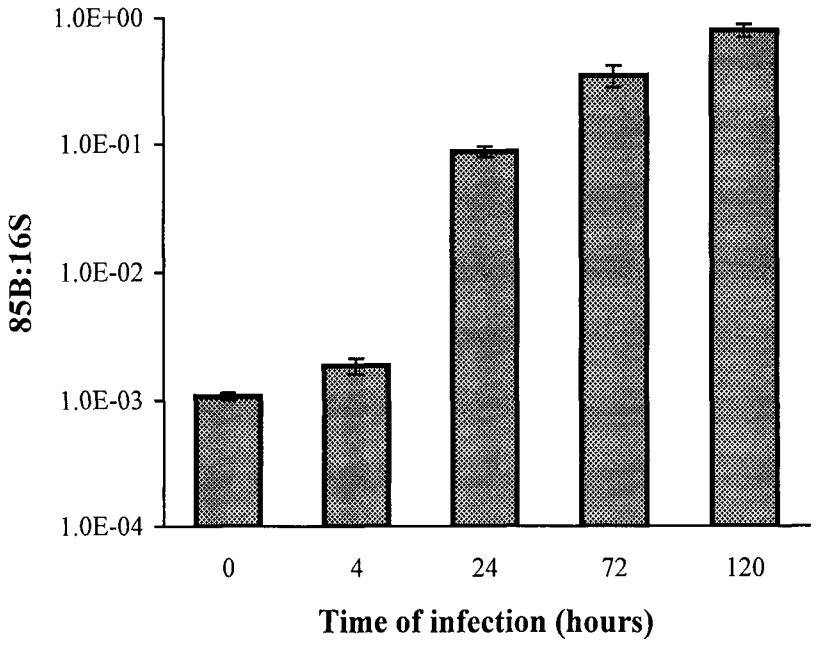


Figure 25. Real-time RT-PCR for kinetics of expression of *M. tuberculosis* 85B:16S ratio: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured for 0–120 hours *in vitro*. Total RNA was extracted and assessed for *M. tuberculosis* 85B:16S ratio. Data represent mean ± SEM of 6 experiments.

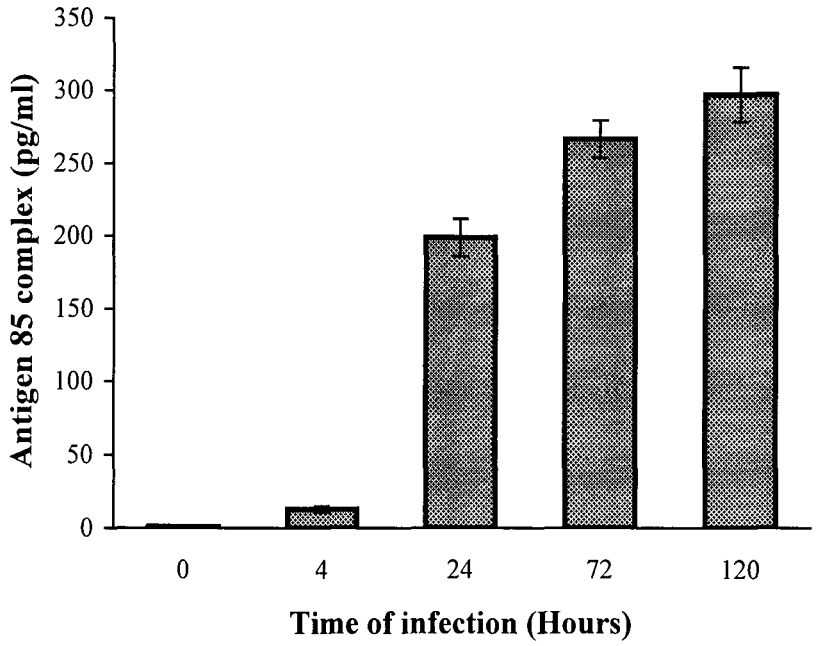


Figure 26. ELISA for kinetics of secreted antigen 85 complex expression: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured for 0–120 hours *in vitro*. Secreted antigen 85 complex was determined in supernatants of monocyte cultures. Data represent mean ± SEM of 6 experiments.

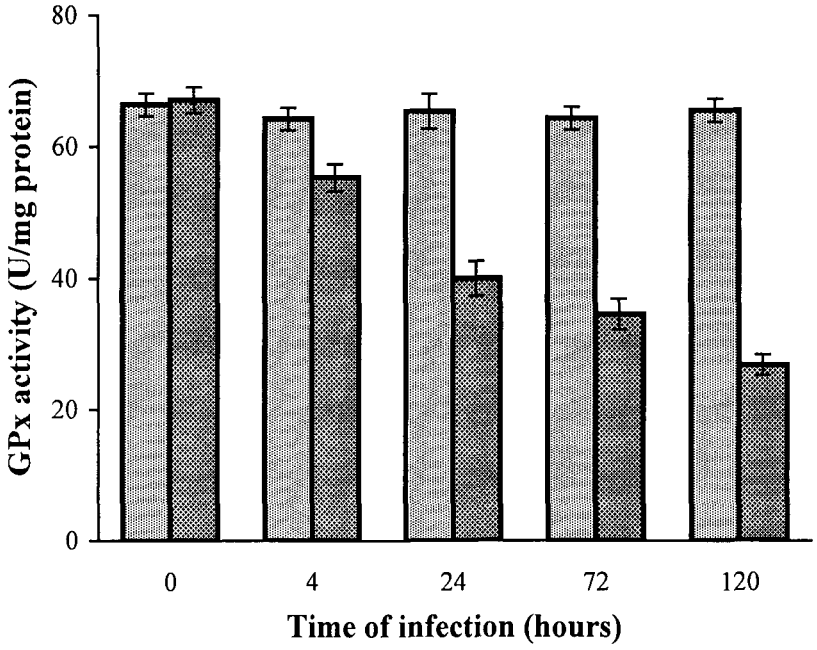


Figure 27. Kinetics of glutathione peroxidase (GPx) activity: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured for 0–120 hours *in vitro*. Some cultures were not infected (white bars) and served as corresponding controls. GPx activity was determined in culture supernatants. Data represent mean ± SEM of 4 experiments.

(D) Modulation Studies of TNF-α and 85B gene expression in *M. tuberculosis*-infected monocytes:

Dose-response effect of exogenous rhTNF-α on host TNF-α and *M. tuberculosis* 85B gene expression in *M. tuberculosis*-infected monocytes:

The above data suggested a role for endogenous TNF-α in auto-induction and in the induction of *M. tuberculosis* 85B gene expression. To further assess the role of TNF-α, in other experiments, exogenous rhTNF-α was added to *M. tuberculosis*-infected monocytes, and then TNF-α and 85B mRNA were assessed. In these experiments, varying doses of rhTNF-α (0.1, 0.2, 2 and 10 ng/ml), or medium alone, were added to *M. tuberculosis*-infected monocytes. Thereafter, evaluation of cultures harvested after 24 hours of infection revealed high magnitude expression of endogenous TNF-α mRNA as a result of *M. tuberculosis* infection in monocytes devoid of exogenous rhTNF-α. In comparison to control infected monocytes, an increase in endogenous TNF-α mRNA by around 1.2 logs ($P<0.001$), 1.5 logs ($P<0.001$), 2.2 logs ($P<0.001$) and 4.2 logs ($P<0.001$) was recorded with treatments of 0.1, 0.2, 2 and 10 ng/ml of rhTNF-α, respectively (Fig. 28). Interestingly, a significant change was seen in 85B:16S ratio after 24 hours of *M. tuberculosis* infection. As evident from Fig. 29, an increase in 85B:16S ratio was observed with the increase in dose of rhTNF-α. Thus, the 85B:16S ratio observed were 0.089, 0.319 ($P<0.01$), 0.375 ($P<0.001$), 0.444 ($P<0.001$) and 0.920 ($P<0.001$), with 0, 0.1, 0.2, 2 and 10 ng/ml of rhTNF-α, respectively. Therefore, we conclude that cell activation by exogenous rhTNF-α induces expression of both TNF-α and *M. tuberculosis* 85B genes in *M. tuberculosis*-infected monocytes.

Effect of sTNFR-I and sTNFR-II on TNF-α and *M. tuberculosis* 85B gene expression in *M. tuberculosis*-infected monocytes:

The above data show that the expression of *M. tuberculosis* 85B is elevated after *M. tuberculosis* infection in monocytes, and correlates with TNF-α expression during the first 24 hours of infection. It showed that *M. tuberculosis*-induced TNF-α in monocyte cultures modulated *M. tuberculosis* 85B gene expression during the first 24 hours. To assess an effect of endogenous TNF-α on *M. tuberculosis* 85B expression, we inhibited TNF-α signalling by using its soluble receptors, TNFR-I and TNFR-II,

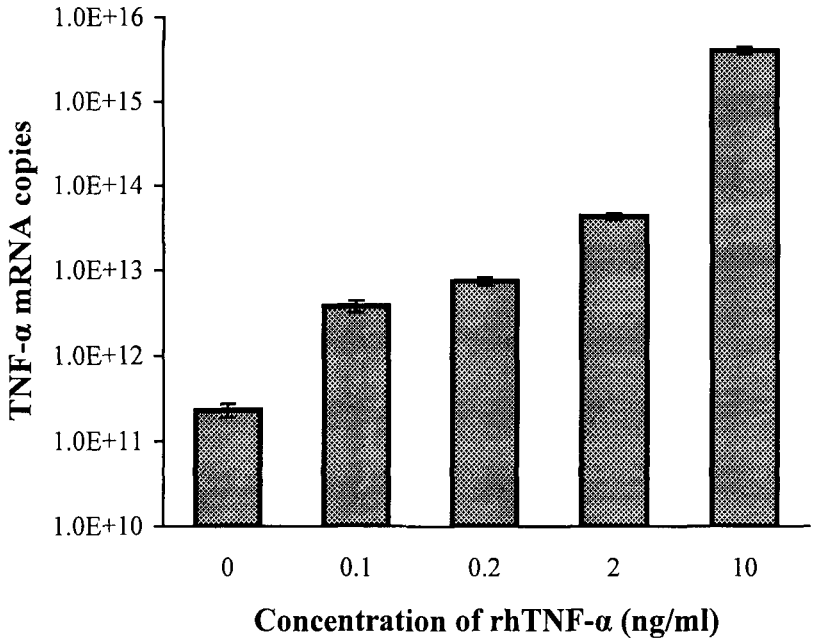


Figure 28. Real-time RT-PCR for effect of exogenous rhTNF-α on expression of TNF-α mRNA: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured with rhTNF-α (0–10 ng/ml) for 24 hours. Total RNA was extracted and assessed for TNF-α mRNA. Data represent mean ± SEM of 6 experiments.

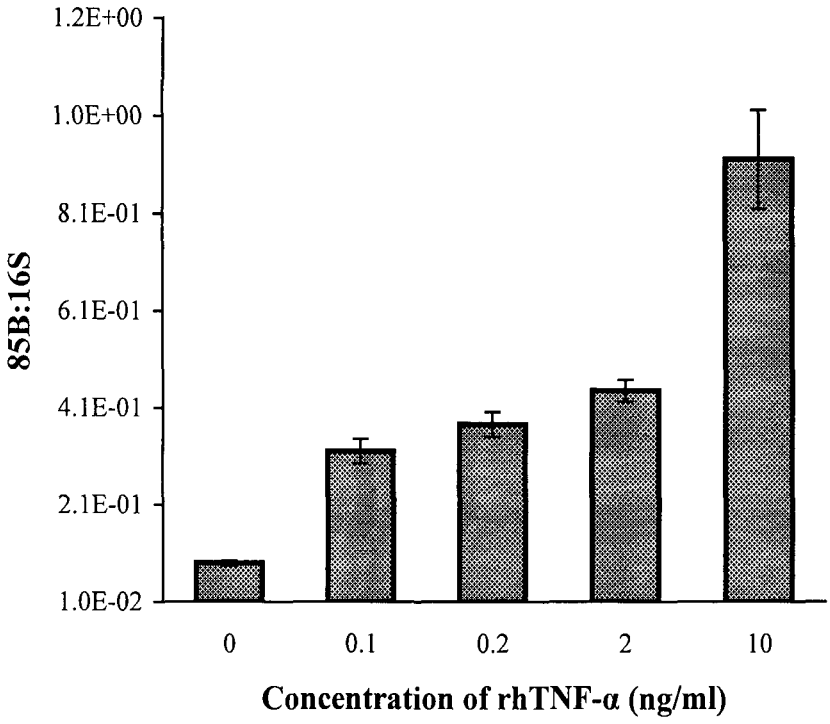


Figure 29. Real-time RT-PCR for effect of exogenous rhTNF-α on expression of *M. tuberculosis* 85B gene: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured with rhTNF-α (0–10 ng/ml) for 24 hours. Total RNA was extracted and assessed for *M. tuberculosis* 85B:16S ratio. Data represent mean ± SEM of 6 experiments.

which bind and inactivate TNF-α. *M. tuberculosis*-infected monocytes were cultured either with sTNFR-I (10 ng/ml) or sTNFR-II (10 ng/ml), or medium alone, for 24 hours. We found that both soluble receptors inhibited TNF-α as well as *M. tuberculosis* 85B mRNA expression in monocytes significantly. In comparison to *M. tuberculosis*-infected monocytes, the infected cells co-cultured with sTNFR-I and sTNFR-II exhibited a suppression in TNF-α mRNA levels by around 5 logs ($P<0.001$) and 3.2 logs ($P<0.001$), respectively (Fig. 30). Furthermore, inhibition of TNF-α by either sTNFR-I or sTNFR-II also decreased *M. tuberculosis* 85B mRNA expression significantly in *M. tuberculosis*-infected monocytes at 24 hours ($P<0.001$ for both) (Fig. 31). Therefore, inhibition of endogenous TNF-α was associated with down-modulation of both TNF-α and *M. tuberculosis* 85B gene expression. The results indicate sTNFR-I to be a stronger inhibitor of TNF-α and *M. tuberculosis* 85B gene expression in *M. tuberculosis*-infected monocytes than sTNFR-II.

Role of NF-κB in *M. tuberculosis*-infected monocytes:

Cellular activation, and thereby induction of TNF-α, is mediated via NF-κB (Toossi et al., 1997; Fan et al., 2002). It has been well documented that TNF-α induced nuclear translocation of NF-κB was inhibited in SN50 peptide-treated human monocytic cell lines as demonstrated in EMSA (Lin et al., 1995). Thus, we employed SN50, an inhibitor of NF-κB, to assess the role of NF-κB in activation of gene expression in *M. tuberculosis*-infected monocytes. SN50 (100 μg/ml) was added to monocytes 3 minutes prior to *M. tuberculosis* infection. Control cultures did not receive SN50. At 24 hours, SN50 suppressed endogenous TNF-α mRNA expression in *M. tuberculosis*-infected monocytes by around 6 logs ($P<0.001$) in comparison to control cultures devoid of SN50 pre-treatment (Fig. 32A). To ensure that cellular inhibition was not non-specific, we compared the effect of SN50 with its inactive analogue, SN50/M at the same concentration. SN50/M did not affect TNF-α mRNA expression ($P>0.05$) (Fig. 32A). Similarly, SN50 was found to decrease the ratio of 85B:16S in *M. tuberculosis*-infected monocytes. This can be best viewed from the data depicted in Fig. 32B, where *M. tuberculosis* 85B:16S ratio was found to be 0.088, 0.0003 ($P<0.001$) and 0.090 ($P>0.05$) for control infected monocytes, infected monocytes challenged with SN50, and infected monocytes challenged with SN50/M, respectively (Fig. 32B). Therefore, it can be

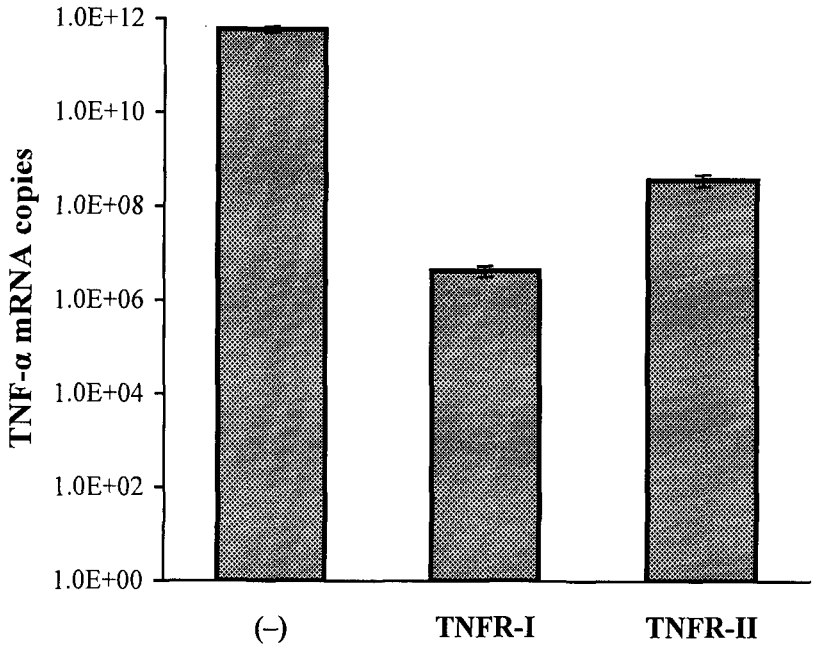


Figure 30. Real-time RT-PCR for effect of soluble (s) TNF receptors (Rs) on expression of TNF-α mRNA: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured with or without (–) 10 ng/ml sTNFR-I and 10 ng/ml sTNFR-II for 24 hours. Total RNA was extracted and assessed for TNF-α mRNA. Data represent mean ± SEM of 6 experiments.

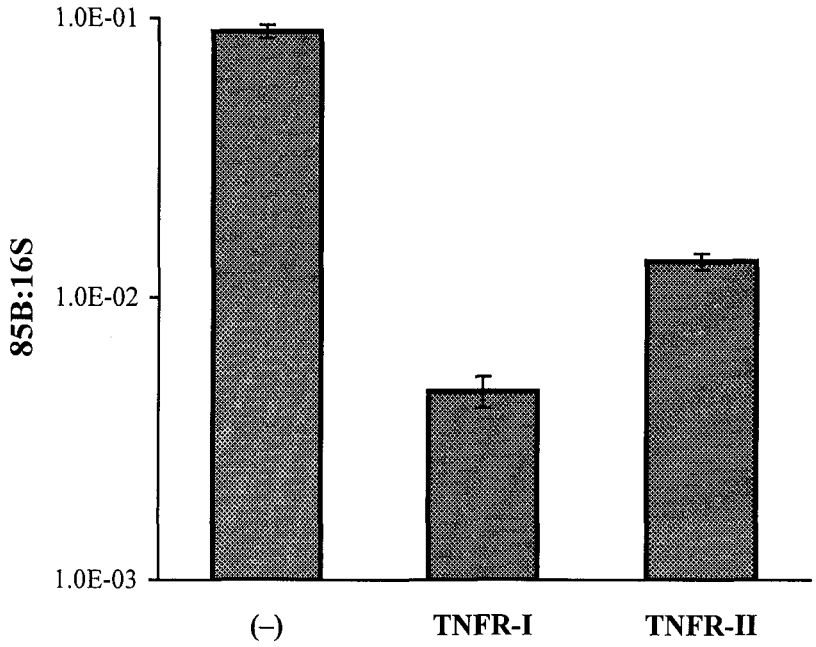


Figure 31. Real-time RT-PCR for effect of soluble (s) TNF receptors (Rs) on expression of *M. tuberculosis* 85B gene: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured with or without (–) 10 ng/ml sTNFR-I and 10 ng/ml sTNFR-II for 24 hours. Total RNA was extracted and assessed for *M. tuberculosis* 85B:16S ratio. Data represent mean ± SEM of 6 experiments.

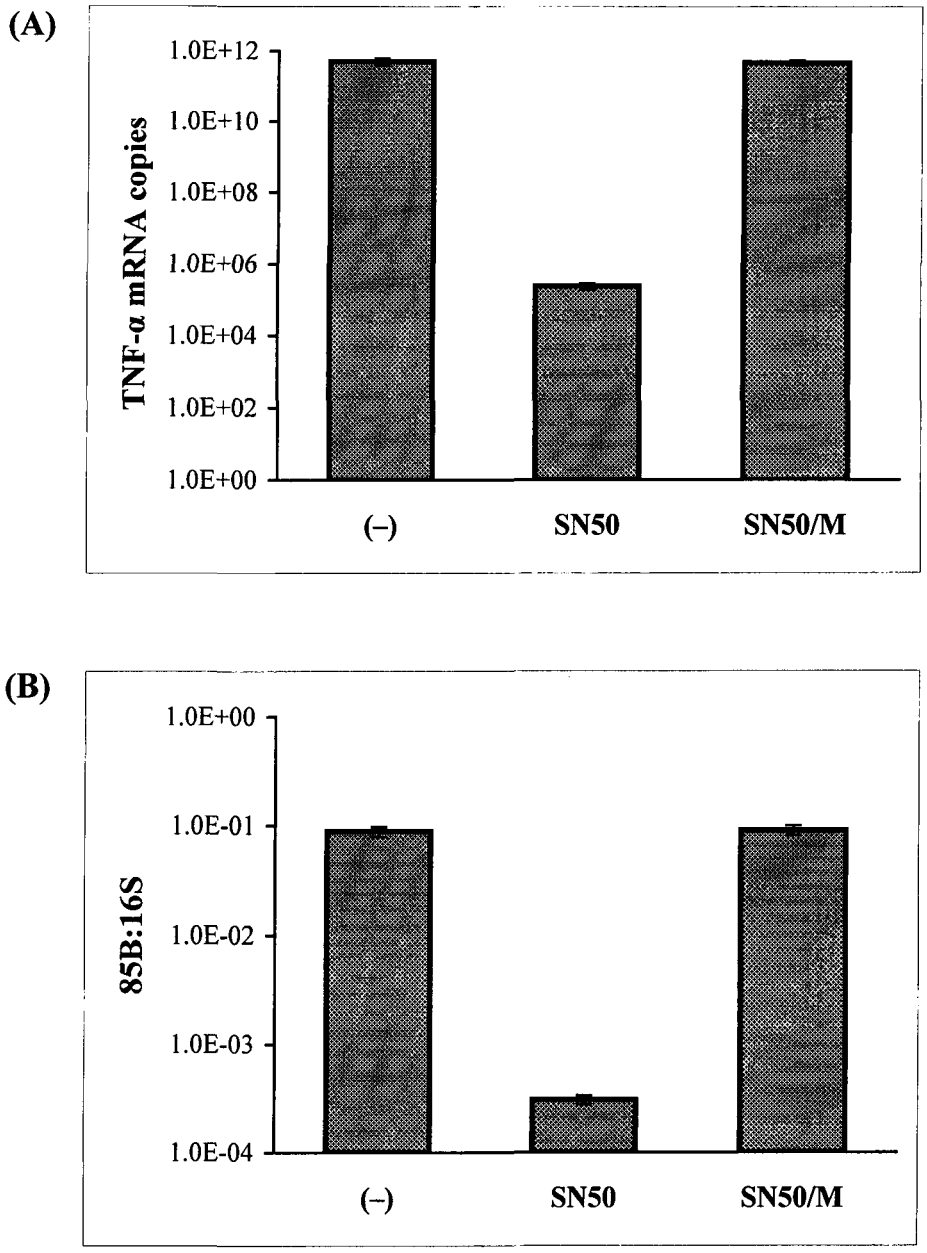


Figure 32. Real-time RT-PCR for effect of inhibition of NFκB on expression of TNF-α and *M. tuberculosis* 85B mRNA: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured with or without (–) 100 μg/ml SN50 and 100 μg/ml SN50/M for 24 hours. Total RNA was extracted and assessed for expression of TNF-α mRNA (A) and *M. tuberculosis* 85B:16S ratio (B). Data represent mean ± SEM of 6 experiments.

inferred that the increased expression of TNF-α and 85B mRNA in *M. tuberculosis*-infected monocytes is mediated mainly via NF-κB.

Role of ROI and RNI in *M. tuberculosis*-infected monocytes:

NAC and NMMA, specific inhibitors of ROS and RNI pathways, and oATP, a known inhibitor of both ROI and RNI pathways, were employed in the present study to assess their effects on TNF-α and 85B mRNA expression in *M. tuberculosis*-infected monocytes. After infection of monocytes with *M. tuberculosis*, NAC (10 mM), NMMA (10 mM), or oATP (10 mM) were added to cultures. Control cultures received medium alone. Antioxidant effects of NAC, NMMA and oATP on TNF-α and 85B gene expression were probed, and it was found that NAC, NMMA and oATP downregulated TNF-α mRNA expression by around 6.2 logs ($P<0.001$), 6.4 logs ($P<0.001$) and 7.1 logs ($P<0.001$), respectively, in cultures harvested after 24 hours of infection (Fig. 33A), as compared to control cultures. Furthermore, evaluation of *M. tuberculosis* 85B:16S ratio revealed similar results, with all three inhibitors decreasing the ratio of 85B:16S by an appreciable magnitude ($P<0.001$ for all), when compared with infected monocytes devoid of any inhibitor (Fig. 33B).

Furthermore, in view of the data obtained above with negative modulators, attempts were also made to probe the effects of positive modulators, namely, SNP, NADPH and NOC-9, on TNF-α and 85B gene expression in *M. tuberculosis*-infected monocytes. As evident from Fig. 34A, in comparison to control infected cultures devoid of any supplements and harvested after 24 hours of infection, *M. tuberculosis*-infected monocytes that were co-cultured with SNP (0.5 μM), NADPH (5 μM) and NOC-9 (300 μM) exhibited an augmentation in TNF-α by around 1 log ($P<0.01$), 3.1 logs ($P<0.001$) and 2.2 logs ($P<0.001$), respectively.

On determining the *M. tuberculosis* 85B:16S ratio for the above cells, an appreciable increase in 85B:16S ratio was observed with the above positive modulators. As illustrated in Fig. 34B, the 85B:16S ratio was 0.043 for control monocytes infected with *M. tuberculosis* that were devoid of any modulator, whereas the 85B:16S ratio was 0.431 ($P<0.001$), 0.737 ($P<0.001$) and 1.072 ($P<0.001$) for infected monocytes co-cultured with SNP, NOC-9 and NADPH, respectively.

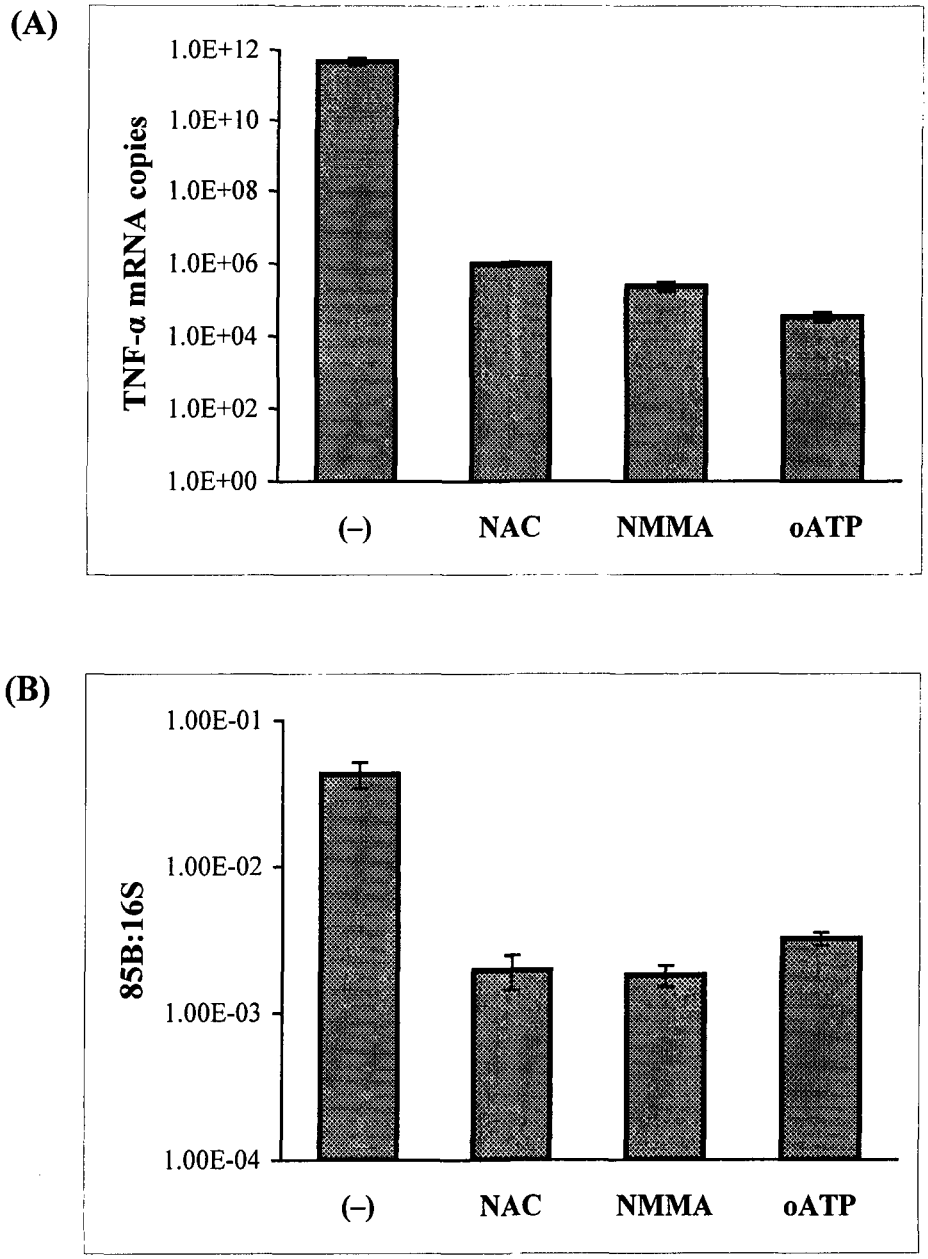


Figure 33. Real-time RT-PCR for inhibition of expression of TNF-α and *M. tuberculosis* 85B mRNA by NAC, NMMA and oATP: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured with or without (–) 10 mM N-acetyl cysteine (NAC), N^G-monomethyl-L-arginine monoacetate (NMMA), or oxidized ATP (oATP) for 24 hours. Total RNA was extracted and assessed for TNF-α mRNA (A) and *M. tuberculosis* 85B:16S ratio (B). Data represent mean ± SEM of 6 experiments.

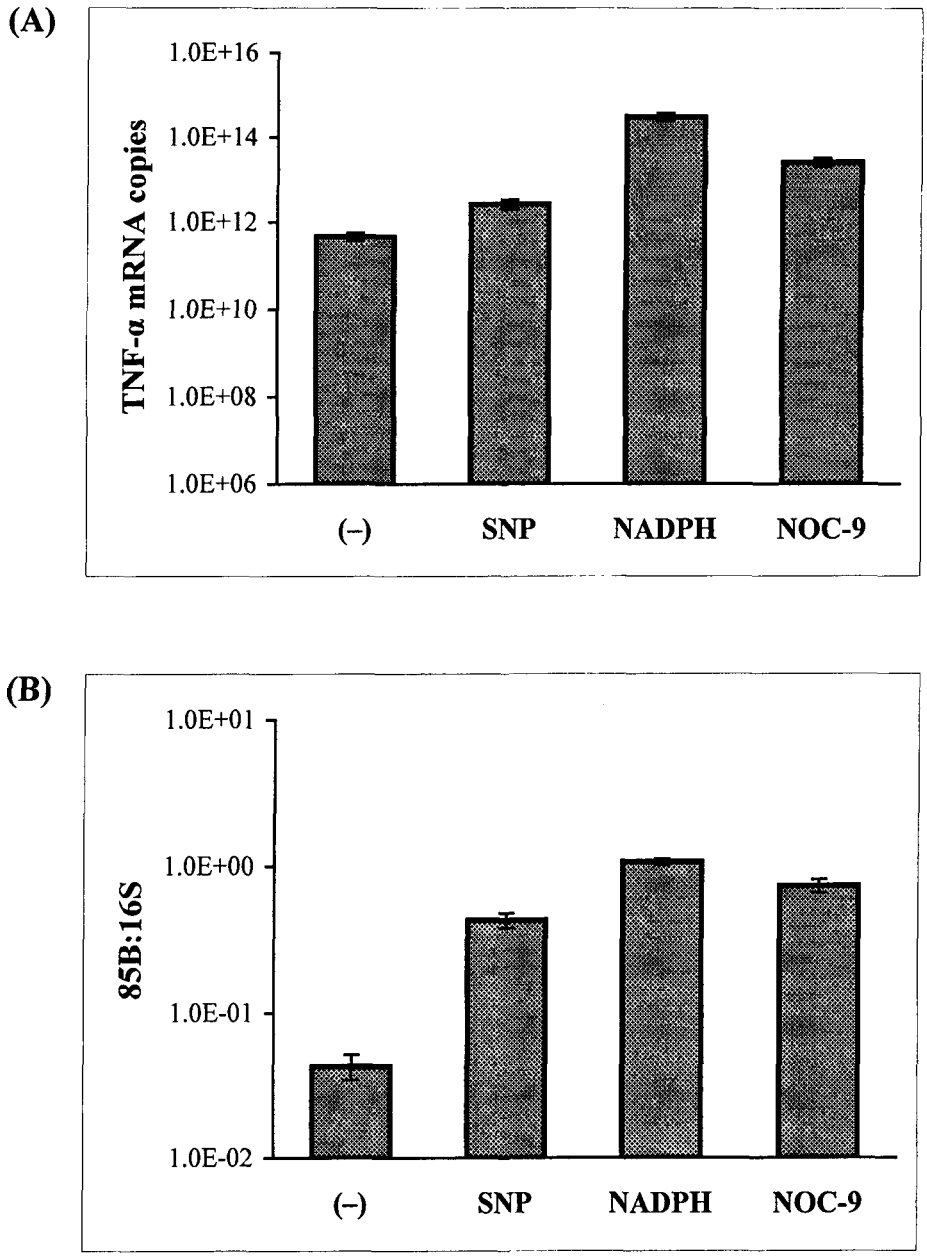


Figure 34. Real-time RT-PCR for induction of expression of TNF-α and *M. tuberculosis* 85B mRNA by SNP, NADPH and NOC-9: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured with or without (–) sodium nitroprusside (SNP; 0.5 μM), NADPH (5 μM), or nonoate-9 (NOC-9; 300 μM) for 24 hours. Total RNA was extracted and assessed for TNF-α mRNA (A) and *M. tuberculosis* 85B:16S ratio (B). Data represent mean ± SEM of 6 experiments.

(E) Modulation Studies of TNF-α and *M. tuberculosis* 85B Expression with the Natural Antioxidant Allicin:

Toxicity assessment of allicin:

Since higher doses of allicin from garlic have previously proven to be toxic by various investigators, the present study employed lower concentrations (0–500 ng/ml), which failed to show any toxic effect on monocytes as revealed by MTT (Fig. 35) or trypan blue exclusion assays. Cell viability is expressed as percent (mean ± SEM) viable cells compared to untreated cells (taken as 100% viable). Also, no effect was observed on housekeeping genes: R18 (18S rRNA) by quantitative real-time RT-PCR (Fig. 36A), or β-actin gene as revealed by RT-PCR (Fig. 36B), thereby indicating that allicin did not non-specifically affect human TNF-α transcription. Also, the effect of various doses of allicin employed in the present study failed to show any toxic or inhibitory effect ($P>0.05$ for all) on the mycobacterial housekeeping gene *M. tuberculosis* 16S rRNA by quantitative real-time RT-PCR in monocytes infected with *M. tuberculosis* and cultured for 24 hours (Fig. 37A). Furthermore, no effect was observed on amplification of *M. tuberculosis* 16S rRNA as revealed by RT-PCR results (Fig. 37B).

Dose-response effect of allicin on TNF-α gene expression:

M. tuberculosis-infected monocytes were co-cultured for 24 hours with varying doses of natural antioxidant allicin. Infected cultures devoid of allicin served as control. The dose-response effect of allicin on TNF-α gene expression was determined by real-time RT-PCR: in comparison to control, a linear downregulation of TNF-α mRNA copy numbers by ~3.2 logs ($P<0.001$), 4.1 logs ($P<0.001$), 7.1 logs ($P<0.001$) and 8.1 logs ($P<0.001$) was recorded with 50, 100, 250 and 500 ng/ml of allicin, respectively (Fig. 38A). This was further substantiated by RT-PCR. The amplification of TNF-α gene in RT-PCR was dose-dependent, where concentrations of 250 and 500 ng/ml allicin proved to be potent inhibitors as is evident from RT-PCR products (Fig. 38B). Thus, allicin potently inhibits the gene expression of pro-inflammatory cytokine TNF-α.

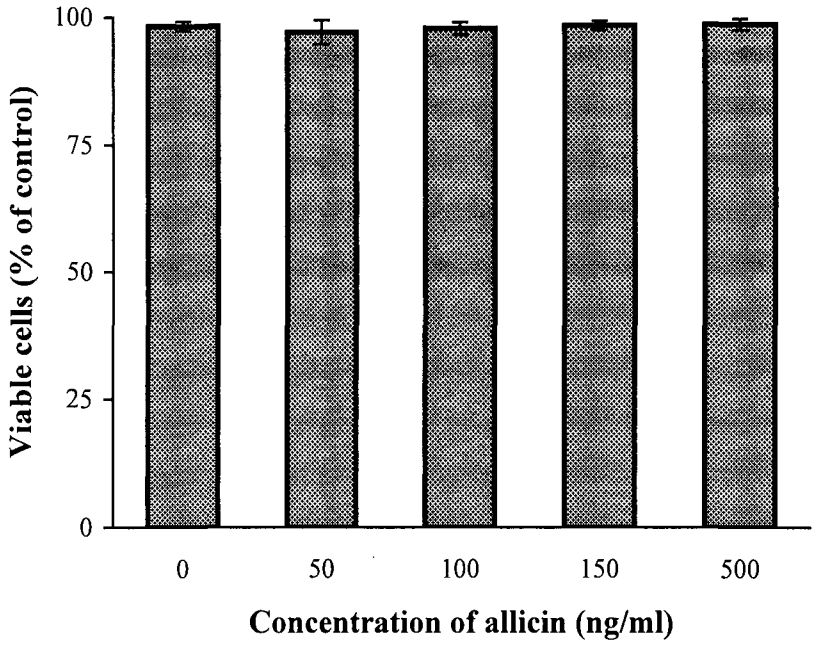


Figure 35. MTT cell viability assay for dose-response effect of allicin (0–500 ng/ml) on *M. tuberculosis*-infected monocytes. Data represents the analysis of three independent experiments in duplicates, expressed as mean viable cells (±SEM) percentage of controls.

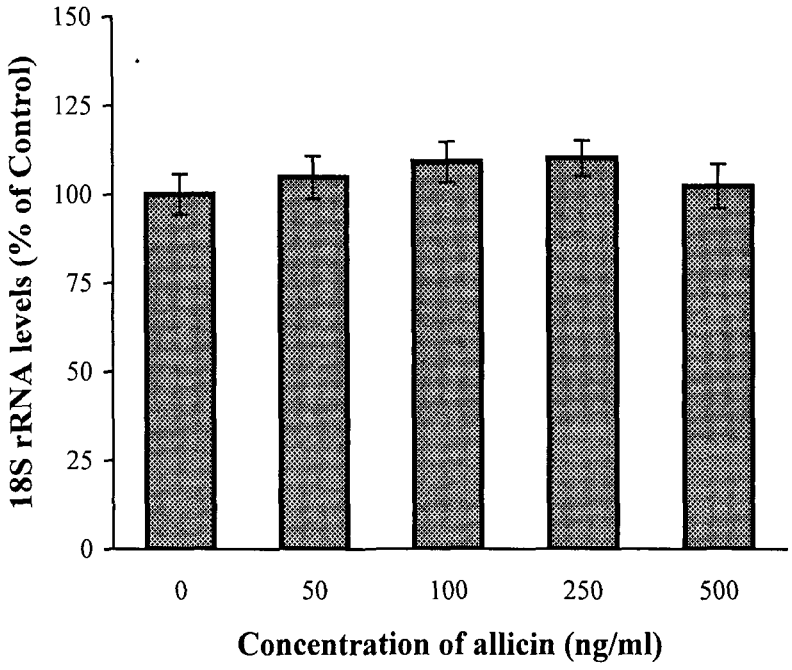


Figure 36A. Real-time RT-PCR for effect of allicin on expression of host housekeeping gene: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured with allicin (0–500 ng/ml) for 24 hours. Total RNA was extracted and assessed for host R18 gene (18S rRNA). Data represent mean ± SEM of 6 experiments.

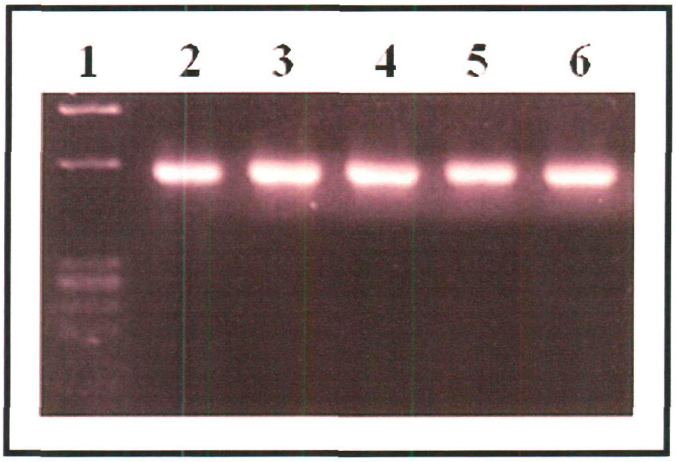


Figure 36B. **RT-PCR for effect of allicin on amplification of β-actin gene: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured with allicin (0–500 ng/ml) for 24 hours. The amplification product for β-actin (514 bp) is shown. Lane (1) DNA ladder; Lanes 2–6 having 0, 50, 100, 250 and 500 ng/ml allicin, respectively.**

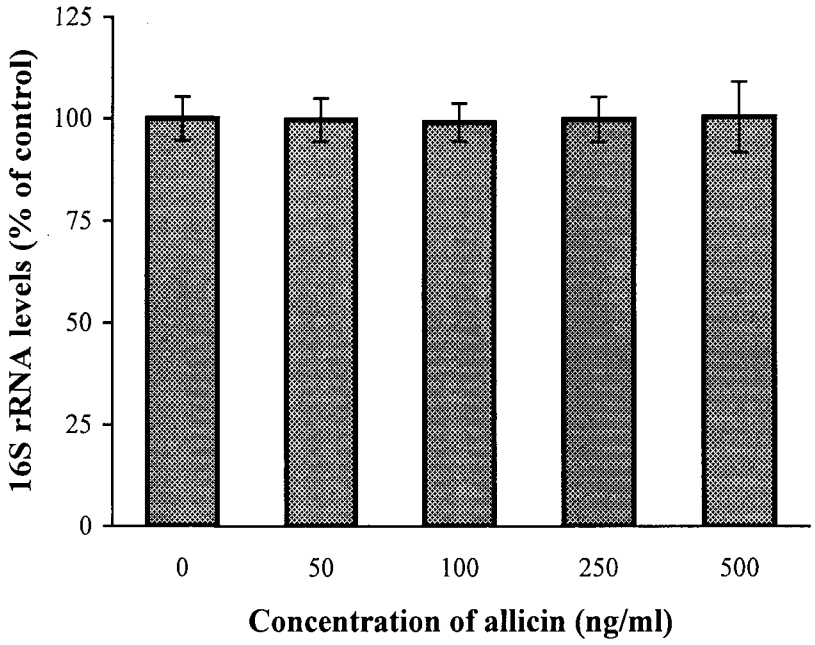


Figure 37A. Real-time RT-PCR for effect of allicin on expression of mycobacterial housekeeping gene: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured with allicin (0–500 ng/ml) for 24 hours. Total RNA was extracted and assessed for mycobacterial 16S rRNA. Data represent mean ± SEM of 6 experiments.

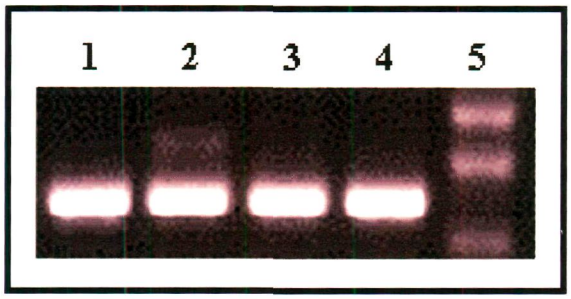


Figure 37B. **RT-PCR for effect of allicin on expression of mycobacterial housekeeping gene: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured with allicin (0–500 ng/ml) for 24 hours. The amplification product for 16S rRNA is shown. Lanes 1–4 having 0, 50, 250 and 500 ng/ml allicin, respectively and Lane (5) DNA ladder.**

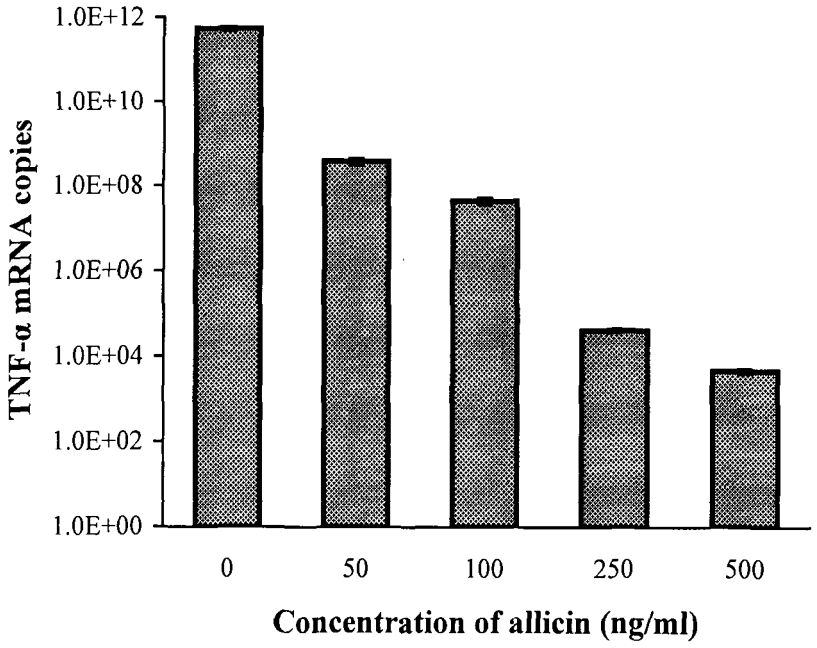


Figure 38A. Real-time RT-PCR for dose-response effect of allicin on expression of TNF-α mRNA: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured with allicin (0–500 ng/ml) for 24 hours. Total RNA was extracted and assessed for TNF-α mRNA. Data represent mean ± SEM of 6 experiments

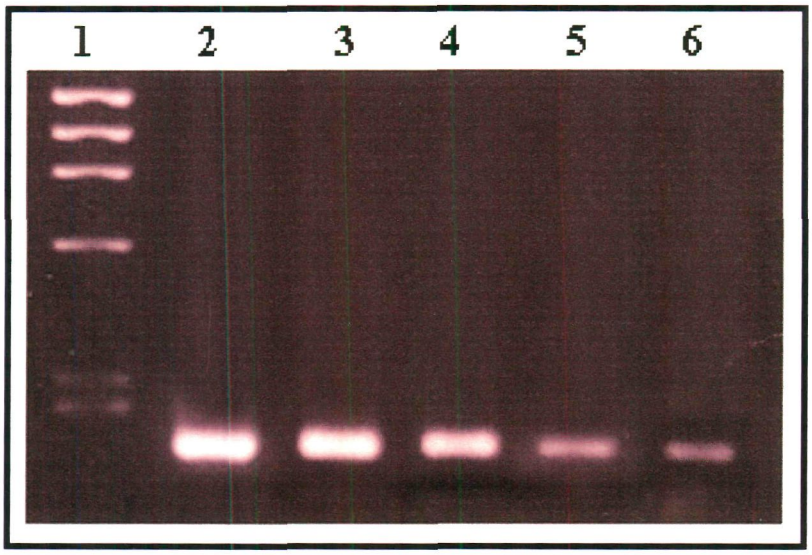


Figure 38B. **RT-PCR for dose-response effect of allicin on amplification of TNF-α mRNA: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured with allicin (0–500 ng/ml) for 24 hours. The amplification product of TNF-α (342 bp) is shown as: Lane (1) DNA ladder; Lanes 2–6 having 0, 50, 100, 250 and 500 ng/ml allicin, respectively.**

Dose-response effect of allicin on soluble TNF-α in *M. tuberculosis*-infected monocyte cultures:

These experiments probed the effect of varying doses of allicin on the expression of soluble TNF-α in 24 hours culture supernatants by ELISA. The suppression in soluble TNF-α expression with varying doses of allicin in 24 hours culture supernatants of *M. tuberculosis*-infected monocytes was found to be dose-dependent, which is evident from the data in Fig. 39, where the concentrations obtained for soluble TNF-α were 187.5, 185.5 ($P>0.05$), 181 ($P>0.05$), 50.5 ($P<0.001$) and 38.7 ($P<0.001$) pg/ml at 0, 50, 100, 250 and 500 ng/ml of allicin, respectively. These results show that at concentrations of 250 and 500 ng/ml, allicin potently inhibits the expression of soluble TNF-α in supernatants of *M. tuberculosis*-infected monocyte cultures. Thus, the doses of 250 and 500 ng/ml proved to be highly potent inhibitory concentrations of allicin.

Dose-response effect of allicin on *M. tuberculosis* 85B gene expression:

Next, we studied the dose-response effect of allicin on the *M. tuberculosis* 85B gene expression in *M. tuberculosis*-infected monocytes that were cultured with or without various doses of allicin for 24 hours. The expression of 85B mRNA was normalized with mycobacterial 16S rRNA and expressed as 85B:16S. Thereafter, the 85B:16S ratio was computed to evaluate the effect of allicin. Fig. 40A clearly shows that the 85B:16S ratio decreased linearly with increase in allicin concentration. The 85B:16S ratio recorded were 0.091, 0.087 ($P>0.05$), 0.012 ($P<0.001$), 0.001 ($P<0.001$) and 0.0002 ($P<0.001$) in *M. tuberculosis*-infected monocytes treated with 0, 50, 100, 250 and 500 ng/ml of allicin, respectively. Similar observations were found in RT-PCR experiments where maximum downregulation in *M. tuberculosis* 85B mRNA expression was observed with 500 ng/ml of allicin (Fig. 40B).

Dose-response effect of allicin on secreted antigen 85 complex in *M. tuberculosis*-infected monocyte cultures:

Thereafter, we probed the dose-response effect of allicin on secreted antigen 85 complex in culture supernatants of *M. tuberculosis*-infected monocytes harvested after 24 hours using ELISA. As illustrated in Fig. 41, the effect of allicin on secreted

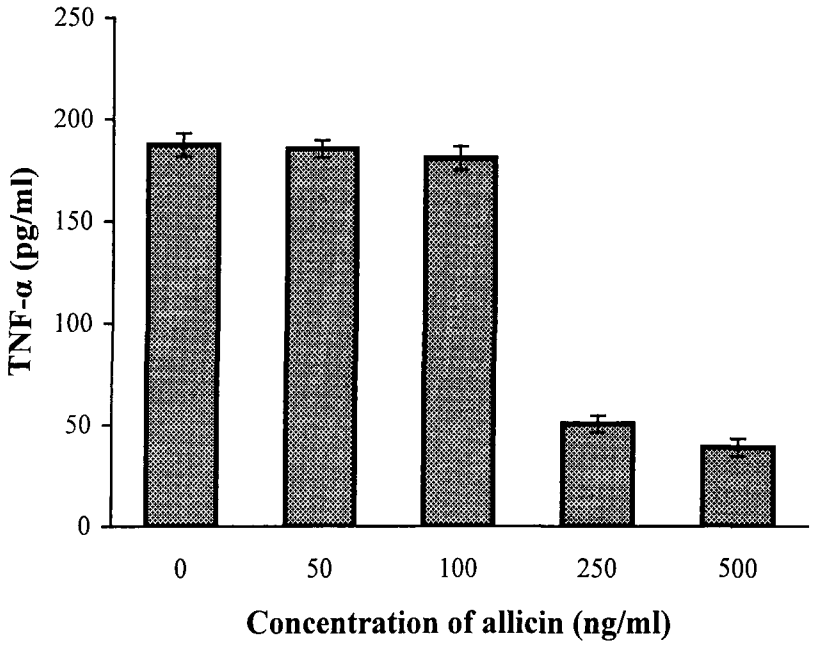


Figure 39. ELISA for dose-response effect of allicin on soluble TNF-α expression: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured with allicin (0–500 ng/ml) for 24 hours. Soluble TNF-α was determined in supernatants of monocyte cultures. Data represent mean ± SEM of 6 experiments.

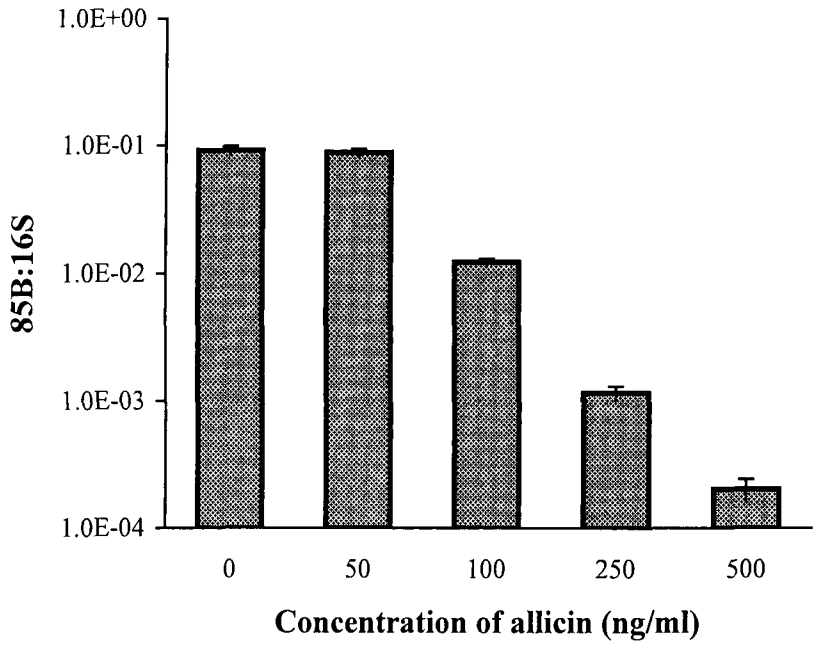


Figure 40A. Real-time RT-PCR for dose-response effect of allicin on expression of *M. tuberculosis* 85B gene: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured with allicin (0–500 ng/ml) for 24 hours. Total RNA was extracted and assessed for *M. tuberculosis* 85B:16S ratio. Data represent mean ± SEM of 6 experiments.

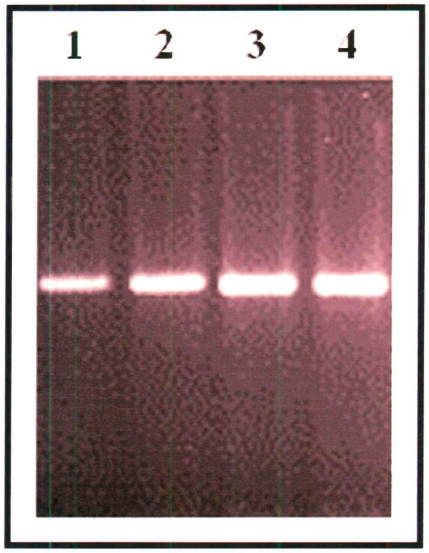


Figure 40B. **RT-PCR for dose-response effect of allicin on amplification of *M. tuberculosis* 85B mRNA: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured with allicin (0–500 ng/ml) for 24 hours. The amplification product of 85B is shown as: Lanes 1–4 having 500, 250, 100 and 0 ng/ml allicin, respectively.**

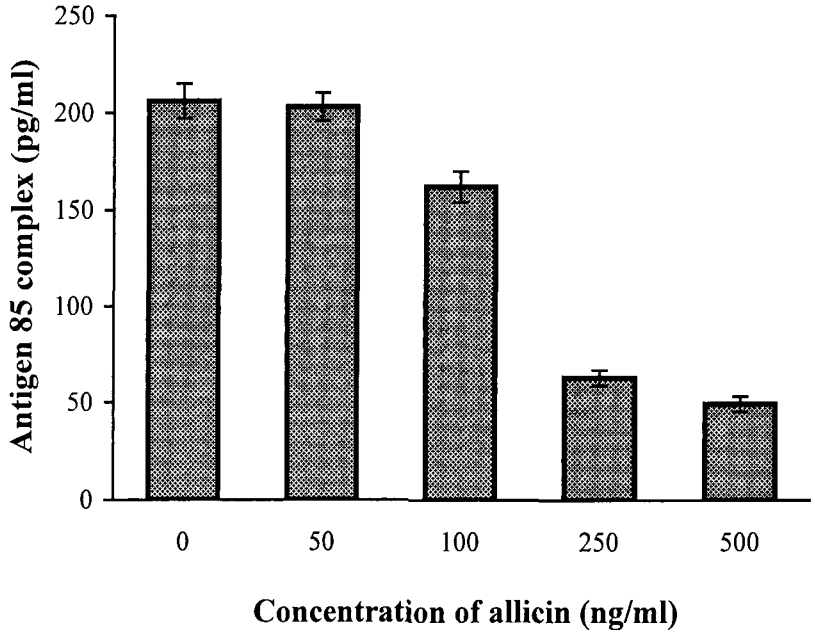


Figure 41. ELISA for dose-response effect of allicin on expression of antigen 85 complex: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured with allicin (0–500 ng/ml) for 24 hours. Secreted antigen 85 complex was determined in supernatants of monocyte cultures. Data represent mean ± SEM of 6 experiments.

antigen 85 complex was found to be dose-dependent. The concentrations of antigen 85 complex secreted in supernatants of infected monocyte cultures at various doses of allicin treatment were recorded as 206.1, 203.2 ($P>0.05$), 162.3 ($P<0.01$), 62.9 ($P<0.001$) and 49.3 ($P<0.001$) pg/ml with 0, 50, 100, 250 and 500 ng/ml of allicin, respectively. Thus, the maximum inhibition in secretion of antigen 85 complex in culture supernatant was observed with 500 ng/ml of allicin.

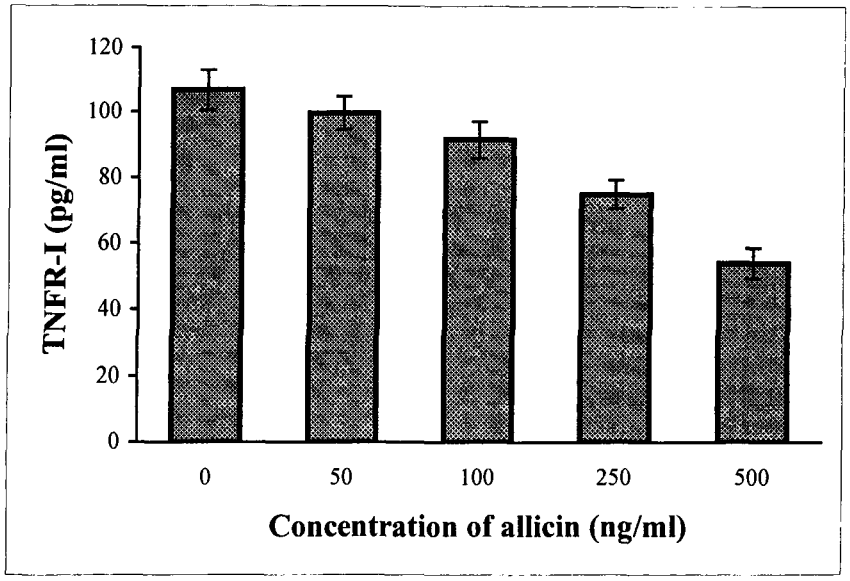
Dose-response effect of allicin on secreted TNFR-I and TNFR-II in *M. tuberculosis*-infected monocyte cultures:

The effect of varying doses of allicin on the levels of secreted soluble TNF receptors, TNFR-I and TNFR-II, in supernatants of *M. tuberculosis*-infected monocyte cultures was determined by ELISA. As is evident from Fig. 42A, the effect of allicin on secreted TNFR-I in supernatants was found to be dose-dependent, where the concentrations of sTNFR-I observed were 106.8, 99.7 ($P<0.05$), 91.7 ($P<0.01$), 75 ($P<0.001$) and 54 ($P<0.001$) pg/ml with 0, 50, 100, 250 and 500 ng/ml of allicin, respectively. A similar trend was observed for sTNFR-II, which is clearly evident from Fig. 42B. The data obtained for sTNFR-II were 158, 154.8 ($P>0.05$), 132.3 ($P<0.01$), 65.7 ($P<0.001$) and 35.7 ($P<0.001$) pg/ml with 0, 50, 100, 250 and 500 ng/ml of allicin, respectively.

Dose-response effect of NAC on TNF-α and 85B gene expression in *M. tuberculosis*-infected monocytes:

We next investigated the dose-response effect of NAC, an ROI inhibitor, on the expression of TNF-α and 85B mRNA. *M. tuberculosis*-infected monocytes were cultured with or without 1, 2, 4, 6, 8 and 10 mM of NAC for 24 hours. Thereafter, the cells were harvested and processed as per experimental design. The inhibitory effect of NAC doses on TNF-α expression is shown in Fig. 43A, where no effect was observed with 1 and 2 mM of NAC. However, with the increase in NAC dose, its inhibitory effect became evident. In comparison to control monocytes, a downregulation of TNF-α mRNA expression by around 0.7 logs ($P<0.01$), 2.7 logs ($P<0.001$), 5 logs ($P<0.001$) and 6 logs ($P<0.001$) was observed with 4, 6, 8 and 10 mM of NAC, respectively (Fig. 43A). Computational analysis of the dose-response data revealed the IC_{50} value of NAC for TNF-α mRNA expression to be 8 mM.

(A)



(B)

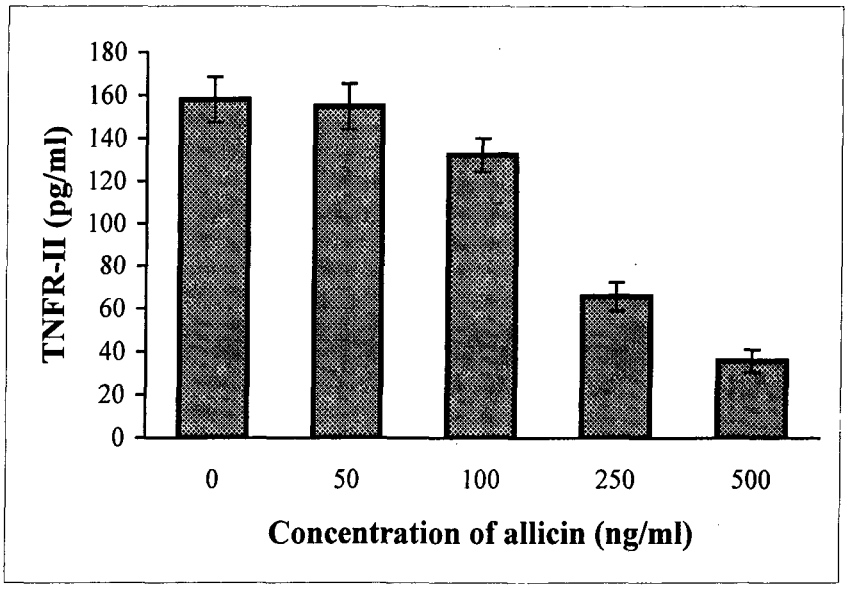
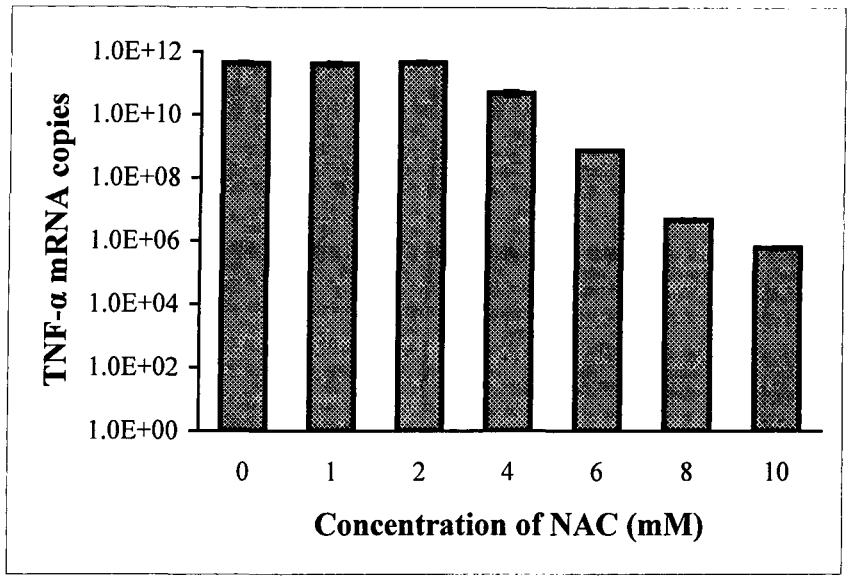


Figure 42. **ELISA for dose-response effect of allicin on soluble TNFR-I and TNFR-II expression: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured with allicin (0–500 ng/ml) for 24 hours. Soluble TNFR-I (A) and TNFR-II (B) were determined in supernatants of monocyte cultures. Data represent mean ± SEM of 6 experiments.**

(A)



(B)

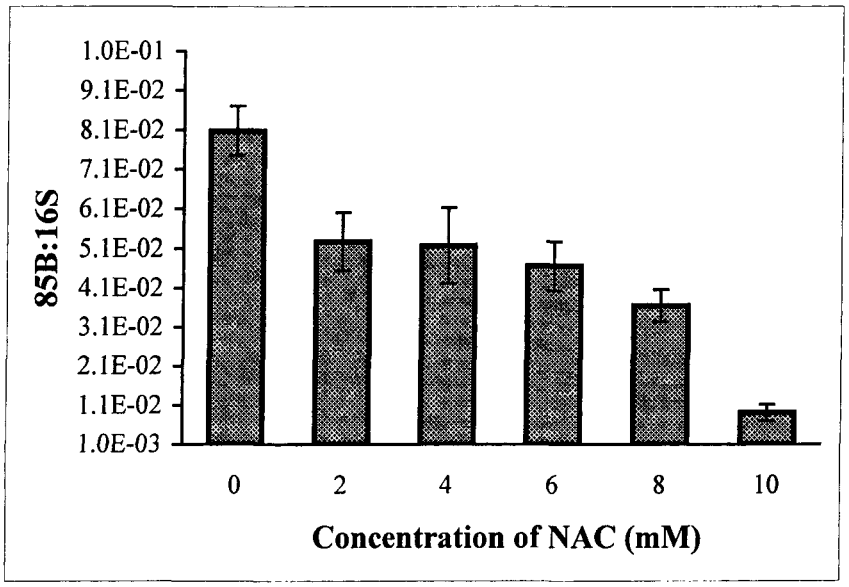


Figure 43. Real-time RT-PCR for dose-response effect of NAC on expression of TNF-α and *M. tuberculosis* 85B mRNA: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured with NAC (0–10 mM) for 24 hours. Total RNA was extracted and assessed for TNF-α mRNA (A) and *M. tuberculosis* 85B:16S ratio (B). Data represent mean ± SEM of 6 experiments.

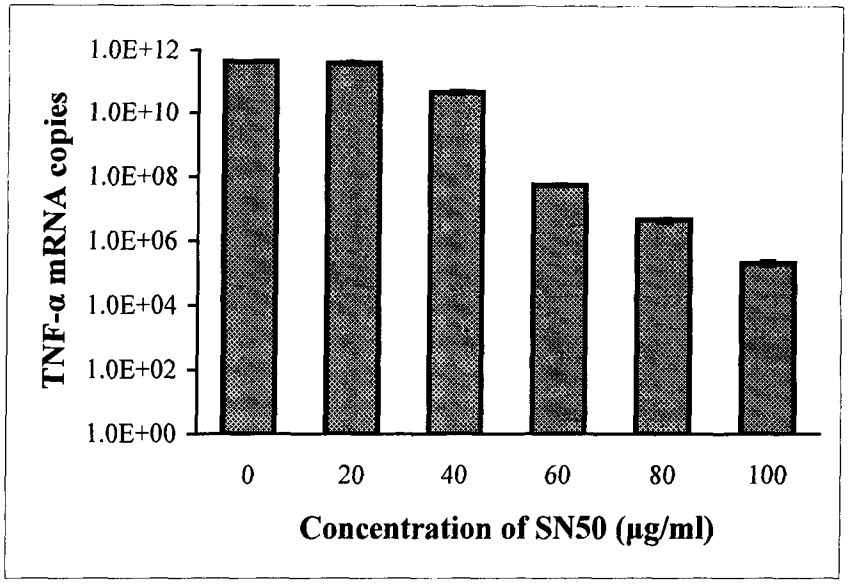
Next, the inhibitory effect of varying doses of NAC on *M. tuberculosis* 85B mRNA expression was probed. As evidenced by data depicted in Fig. 43B, the 85B:16S ratio was found to be dose-dependently modulated. *M. tuberculosis* 85B:16S ratio were recorded as 0.081, 0.053 ($P<0.05$), 0.052 ($P<0.05$), 0.046 ($P<0.01$), 0.036 ($P<0.001$) and 0.009 ($P<0.001$) with 0, 2, 4, 6, 8 and 10 mM of NAC, respectively (Fig. 43B). Therefore, 10 mM concentration of NAC was found to be most effective in suppressing TNF-α and *M. tuberculosis* 85B mRNA expression in *M. tuberculosis*-infected monocytes at 24 hours of infection.

Dose-response effect of SN50 on TNF-α and 85B gene expression in *M. tuberculosis*-infected monocytes:

Furthermore, an attempt was made to study the dose-response effect of NF-κB inhibitor, SN50 on the expression of TNF-α and 85B mRNA. For this, *M. tuberculosis*-infected monocytes were cultured with or without 20, 40, 60, 80 and 100 μg/ml of SN50. Thereafter, the cells were harvested and subjected to analysis for the determination of TNF-α and 85B mRNA. The inhibitory effect of SN50 doses on TNF-α expression is shown in Fig. 44A, where the downregulation of TNF-α mRNA expression by SN50 was found to be dose-dependent. There was negligible change with 20 μg/ml of SN50, but TNF-α mRNA expression was found downregulated by ~1 log ($P<0.01$), 4 logs ($P<0.001$), 5 logs ($P<0.001$) and 6 logs ($P<0.001$), with 40, 60, 80 and 100 μg/ml of SN50, respectively (Fig. 44A). Computational analysis of the dose-response data revealed the IC_{50} value of SN50 for TNF-α mRNA expression to be 90 μg/ml.

Thereafter, the inhibitory effect of varying doses of SN50 on *M. tuberculosis* 85B mRNA expression was probed. Fig. 44B shows the 85B:16S ratio, which was found to be dose-dependent. *M. tuberculosis* 85B:16S ratio recorded were 0.081, 0.046 ($P<0.02$), 0.029 ($P<0.001$), 0.003 ($P<0.001$), 0.002 ($P<0.001$) and 0.0004 ($P<0.001$) with 0, 20, 40, 60, 80 and 100 μg/ml of SN50, respectively (Fig. 44B). Therefore, 100 μg/ml concentration of SN50 was found to be most effective in downregulating the expression of TNF-α and *M. tuberculosis* 85B mRNA in *M. tuberculosis*-infected monocytes at 24 hours of infection.

(A)



(B)

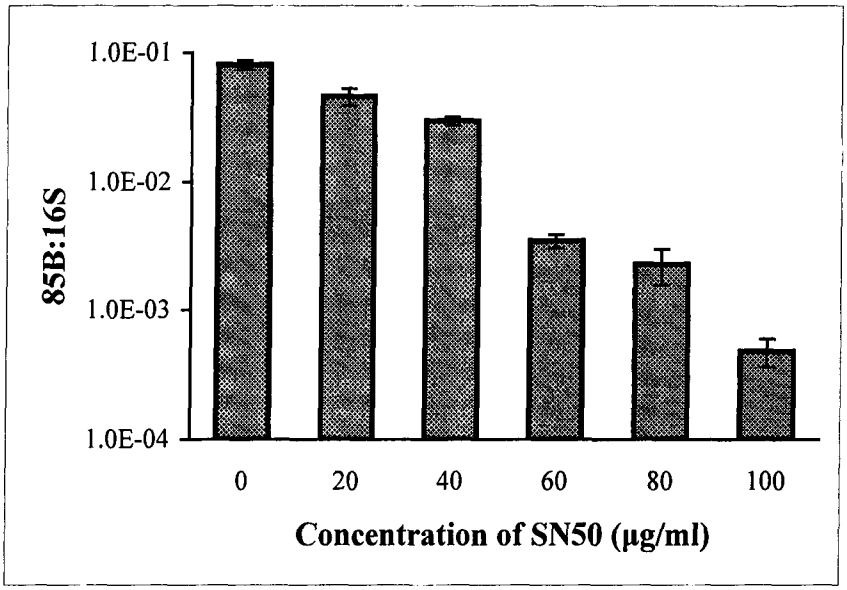


Figure 44. Real-time RT-PCR for dose-response effect of SN50 on expression of TNF-α and *M. tuberculosis* 85B mRNA: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured with SN50 (0–100 μg/ml) for 24 hours. Total RNA was extracted and assessed for TNF-α mRNA (A) and *M. tuberculosis* 85B:16S ratio (B). Data represent mean ± SEM of 6 experiments.

Comparative study of modulatory effects induced by anti-TNF-α antibody versus allicin on TNF-α expression in *M. tuberculosis*-infected monocytes:

In continuation with the above experiments, an attempt was also made to have an insight on the comparative inhibitory effect of anti-TNF-α antibody versus allicin, on endogenous TNF-α mRNA expression. As evident from Fig. 45A, in comparison to control uninfected monocytes, endogenous TNF-α mRNA in infected monocytes was found augmented to the order of ~10.1 logs ($P<0.001$), and was further enhanced by ~4 logs ($P<0.05$) with exogenous rhTNF-α. The effect of exogenous rhTNF-α was neutralized substantially by anti-TNF-α antibody or 500 ng/ml allicin, as evident from suppression in endogenous TNF-α mRNA expression, between 8.1 – 8.3 logs ($P<0.01$). No effect was observed in uninfected monocytes that were either treated or untreated with 500 ng/ml of allicin for 24 hours. A suppression in endogenous TNF-α mRNA in *M. tuberculosis*-infected monocytes by ~6.3 logs ($P<0.001$) and ~5.2 logs ($P<0.001$) was observed with 500 ng/ml allicin and 10 ng/ml anti-TNF-α antibody, respectively (Fig. 45A). A similar pattern was observed with RT-PCR products (Fig. 45B). Thus, the data is indicative of TNF-α involvement in positive auto-regulation, and also indicates that allicin proves to be a potent inhibitor of pro-inflammatory cytokine TNF-α, like anti-TNF-α antibody.

After ascertaining the modulatory effects on TNF-α mRNA expression, an attempt was also made to probe the comparative effects of anti-TNF-α antibody and allicin on secreted soluble TNF-α in culture supernatants of *M. tuberculosis*-infected monocytes using ELISA. As evident from Fig. 46, supernatants of monocytes alone and of those co-cultured with 500 ng/ml allicin, but devoid of any *M. tuberculosis* infection, showed negligible or no expression of sTNF-α. On the other hand, 189 pg/ml of sTNF-α was recorded in supernatants of monocytes infected with *M. tuberculosis*. Upon co-culturing *M. tuberculosis*-infected monocytes with 10 ng/ml exogenous rhTNF-α, the sTNF-α level increased to 232.5 pg/ml ($P<0.001$). Addition of 10 ng/ml of anti-TNF-α antibody or 500 ng/ml of allicin to rhTNF-α (10 ng/ml)-challenged *M. tuberculosis*-infected monocytes showed a decrease in sTNF-α levels to 154 pg/ml ($P<0.001$) and 113 pg/ml ($P<0.001$), respectively. A further suppression in

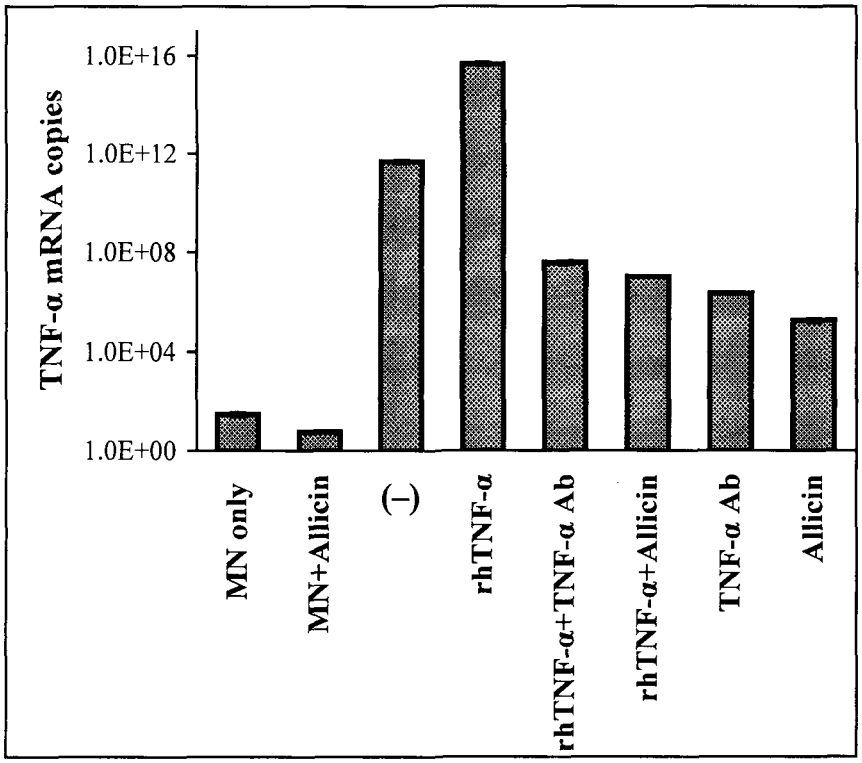


Figure 45A. Real-time RT-PCR for modulation of TNF-α mRNA expression. Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell). Some cultures were not infected (MN only and MN+Allicin) and served as controls. Cultures then received either rhTNF-α (10 ng/ml) or rhTNF-α (10 ng/ml) + anti-TNF-α Abs (10 ng/ml) or rhTNF-α (10 ng/ml) + allicin (500 ng/ml) or anti-TNF-α Abs (10 ng/ml) or allicin (500 ng/ml) or media alone (–) for 24 hours. Total RNA was extracted and assessed for TNF-α mRNA. Data represent mean ± SEM of 6 experiments.

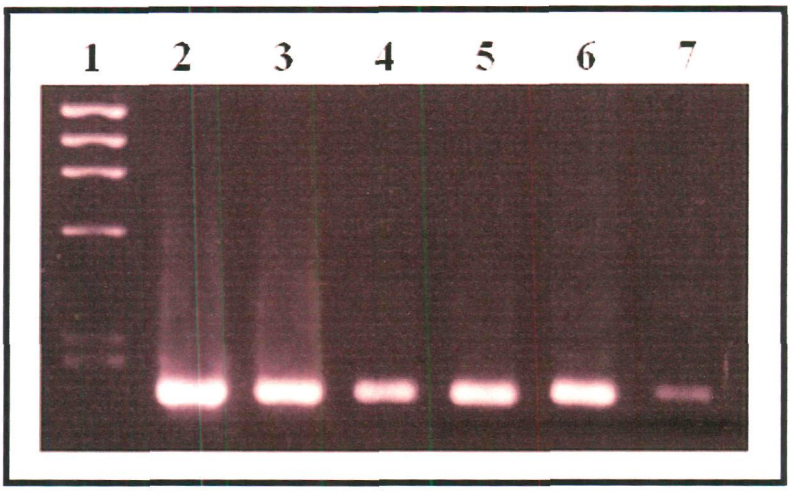


Figure 45B. **RT-PCR for modulation of TNF-α mRNA amplification: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) and cultured for 24 hours. The amplification product of TNF-α is shown as: Lanes (1) DNA ladder, (2) rhTNF-α (10 ng/ml), (3) Infected monocytes, (4) anti-TNF-α Abs (10 ng/ml), (5) rhTNF-α (10 ng/ml) + allicin (500 ng/ml), (6) rhTNF-α (10 ng/ml) + anti-TNF-α Abs (10 ng/ml) and (7) Allicin (500 ng/ml).**

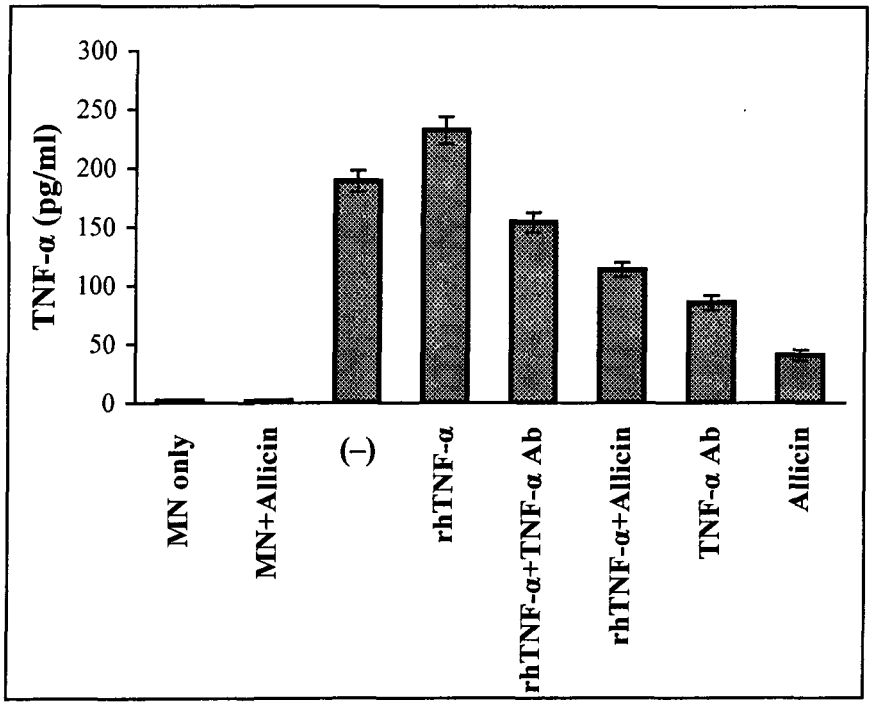


Figure 46. **ELISA for modulation of soluble TNF-α expression: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell). Some cultures were not infected (MN only and MN+Allicin) and served as controls. Cultures then received either rhTNF-α (10 ng/ml) or rhTNF-α (10 ng/ml) + anti-TNF-α Abs (10 ng/ml) or rhTNF-α (10 ng/ml) + allicin (500 ng/ml) or anti-TNF-α Abs (10 ng/ml) or allicin (500 ng/ml) or media alone (–) for 24 hours. Soluble TNF-α was determined in supernatants of monocyte cultures. Data represent mean ± SEM of 6 experiments.**

sTNF-α levels was observed in supernatants of *M. tuberculosis*-infected monocytes by anti-TNF-α antibody and allicin, which was to the order of 85.5 pg/ml ($P<0.001$) and 40.7 pg/ml ($P<0.001$), respectively (Fig. 46). Thus, the data obtained indicate that 500 ng/ml of allicin has a potent inhibitory effect on soluble TNF-α in culture supernatants of *M. tuberculosis*-infected monocytes.

Comparative modulatory effects of anti-TNF-α antibody versus allicin on *M. tuberculosis* 85B expression in *M. tuberculosis*-infected monocytes:

We also investigated the modulatory effects of anti-TNF-α antibody versus allicin on *M. tuberculosis* 85B mRNA expression as well as on secreted soluble *M. tuberculosis* antigen 85 complex. The data depicted in Fig. 47 illustrates the comparative effects on *M. tuberculosis* 85B mRNA expression. In comparison to *M. tuberculosis*-infected monocytes, 85B:16S ratio was significantly reduced by allicin. The 85B:16S ratio was recorded as 0.088 for *M. tuberculosis*-infected monocytes that were devoid of any inhibitor. On the contrary, in the presence of 10 ng/ml anti-TNF-α antibody and 500 ng/ml allicin, the 85B:16S ratio was reduced to the order of 0.002 ($P<0.001$) and 0.0002 ($P<0.001$), respectively (Fig. 47). The results thus indicate the potent inhibitory effect of allicin on the 85B mRNA expression, similar to anti-TNF-α antibody.

Next we probed the comparative inhibitory effects of anti-TNF-α antibody versus allicin on secreted soluble *M. tuberculosis* antigen 85 complex in culture supernatants of monocytes infected with *M. tuberculosis* by ELISA. The data clearly show a higher degree of suppression induced by 500 ng/ml of allicin, than by anti-TNF-α antibody, in secreted antigen 85 complex levels in culture supernatants of *M. tuberculosis*-infected monocytes. The level of secreted antigen 85 complex in supernatants of *M. tuberculosis*-infected monocyte cultures was found to be 204.7 pg/ml, whereas in the presence of 10 ng/ml anti-TNF-α antibody and 500 ng/ml allicin, it was reduced to 91.9 pg/ml ($P<0.001$) and 48.7 pg/ml ($P<0.001$), respectively (Fig. 48).

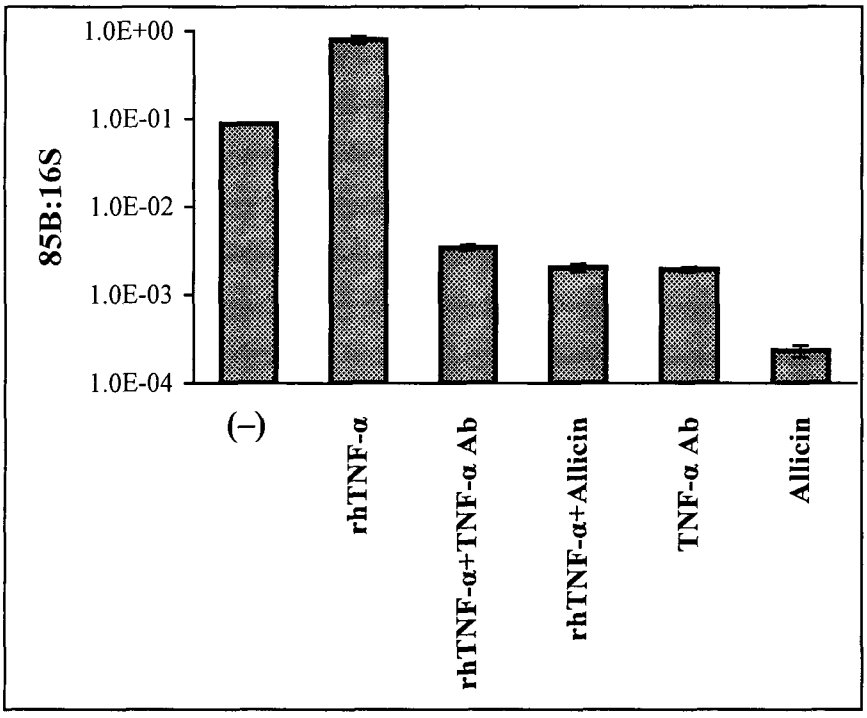


Figure 47. Real-time RT-PCR for modulation of *M. tuberculosis* 85B gene expression: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell). Cultures then received either rhTNF-α (10 ng/ml) or rhTNF-α (10 ng/ml) + anti-TNF-α Abs (10 ng/ml) or rhTNF-α (10 ng/ml) + allicin (500 ng/ml) or anti-TNF-α Abs (10 ng/ml) or allicin (500 ng/ml) or media alone (–) for 24 hours. Total RNA was extracted and assessed for *M. tuberculosis* 85B:16S ratio. Data represent mean ± SEM of 6 experiments.

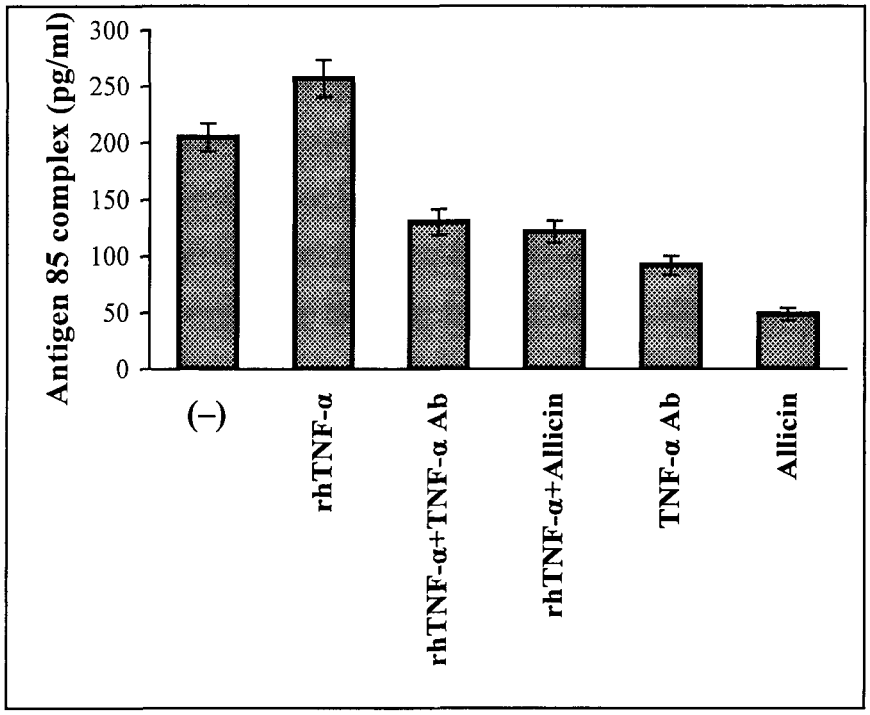


Figure 48. ELISA for modulation of expression of secreted antigen 85 complex: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell). Cultures then received either rhTNF-α (10 ng/ml) or rhTNF-α (10 ng/ml) + anti-TNF-α Abs (10 ng/ml) or rhTNF-α (10 ng/ml) + allicin (500 ng/ml) or anti-TNF-α Abs (10 ng/ml) or allicin (500 ng/ml) or media alone (–). Antigen 85 complex was determined in culture supernatants at 24 hours. Data represent mean ± SEM of 6 experiments.

Comparative modulatory effects of anti-TNF-α antibody versus allicin on secreted soluble TNFR-I and TNFR-II in *M. tuberculosis*-infected monocyte cultures:

We also probed the comparative modulatory effects of anti-TNF-α antibody verses allicin on expression of secreted soluble TNFR-I and TNFR-II using ELISA. As illustrated in Fig. 49, the level of secreted TNFR-I in supernatants of *M. tuberculosis*-infected monocyte cultures was found to be 105 pg/ml, whereas on addition of 10 ng/ml rhTNF-α, it was augmented to 161 pg/ml ($P<0.001$). On the other hand, in the presence of 10 ng/ml anti-TNF-α antibody and 500 ng/ml allicin, sTNFR-I was reduced to 69 pg/ml ($P<0.001$) and 54.7 pg/ml ($P<0.001$), respectively, in *M. tuberculosis*-infected monocyte cultures. Similarly, as evident from Fig. 50, level of secreted TNFR-II in supernatants of *M. tuberculosis*-infected monocyte cultures at 24 hours was recorded as 154 pg/ml. On addition of 10 ng/ml rhTNF-α, it was increased to the order of 208 pg/ml ($P<0.001$). On the contrary, when *M. tuberculosis*-infected monocytes were cultured for 24 hours with 10 ng/ml anti-TNF-α antibody and 500 ng/ml allicin, the level of secreted TNFR-II in supernatants was reduced to 75.5 pg/ml ($P<0.001$) and 30 pg/ml ($P<0.001$), respectively. Thus, the results obtained indicate that allicin is a potent suppressor of secreted TNFR-I and TNFR-II expression, like anti-TNF-α antibody.

Comparative modulatory effects of various inhibitors versus allicin on TNF-α and *M. tuberculosis* 85B mRNA expression in *M. tuberculosis*-infected monocytes:

Here, we investigated the comparative antioxidant effects of NAC versus allicin on TNF-α and *M. tuberculosis* 85B mRNA expression in *M. tuberculosis*-infected monocytes at 24 hours of infection. Comparative antioxidant effect of NAC versus allicin on TNF-α gene expression is shown in Fig. 51, where 10 mM NAC and 500 ng/ml allicin downregulated TNF-α mRNA expression by around 5.5 logs ($P<0.001$) and 6.3 logs ($P<0.001$), respectively, as compared to control *M. tuberculosis*-infected monocytes. Thus, allicin proved to be around 0.8 logs more effective than NAC. The IC_{50} values for TNF-α inhibition in our system, by allicin and NAC were calculated to be 170 ng/ml and 1.3 mg/ml, respectively.

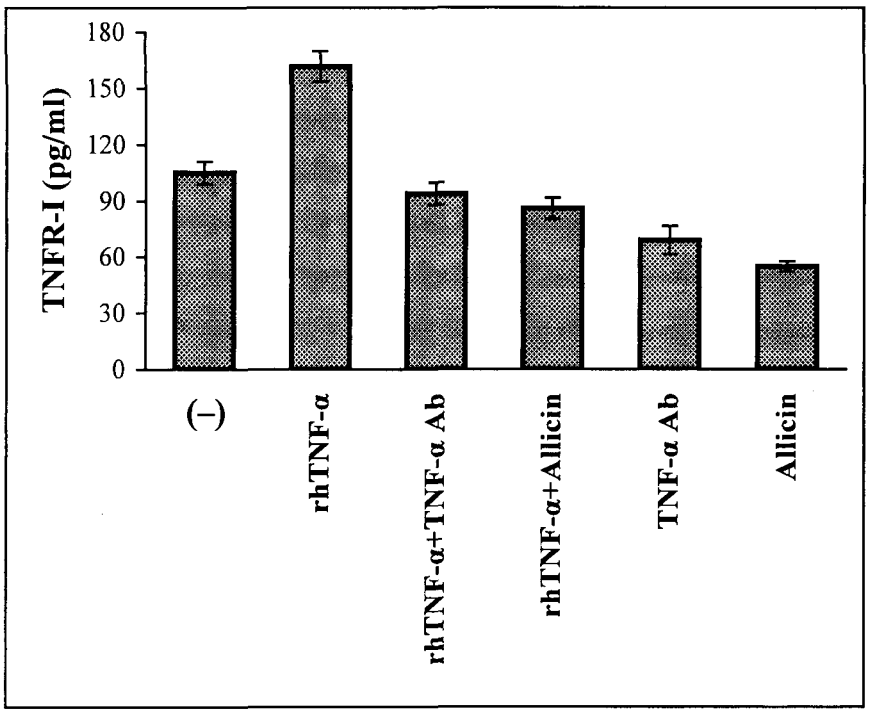


Figure 49. ELISA for modulation of soluble TNFR-I expression: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell). Cultures then received either rhTNF-α (10 ng/ml) or rhTNF-α (10 ng/ml) + anti-TNF-α Abs (10 ng/ml) or rhTNF-α (10 ng/ml) + allicin (500 ng/ml) or anti-TNF-α Abs (10 ng/ml) or allicin (500 ng/ml) or media alone (–) for 24 hours. Soluble TNFR-I was determined in supernatants of monocyte cultures. Data represent mean ± SEM of 6 experiments.

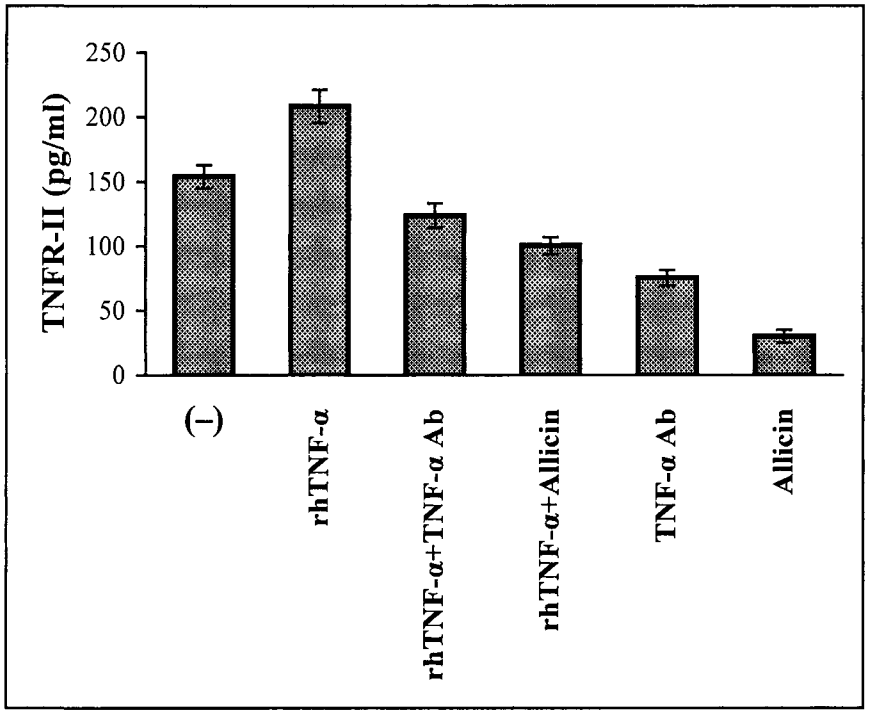


Figure 50. ELISA for modulation of soluble TNFR-II expression: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell). Cultures then received either rhTNF-α (10 ng/ml) or rhTNF-α (10 ng/ml) + anti-TNF-α Abs (10 ng/ml) or rhTNF-α (10 ng/ml) + allicin (500 ng/ml) or anti-TNF-α Abs (10 ng/ml) or allicin (500 ng/ml) or media alone (–) for 24 hours. Soluble TNFR-II was determined in supernatants of monocyte cultures. Data represent mean ± SEM of 6 experiments.

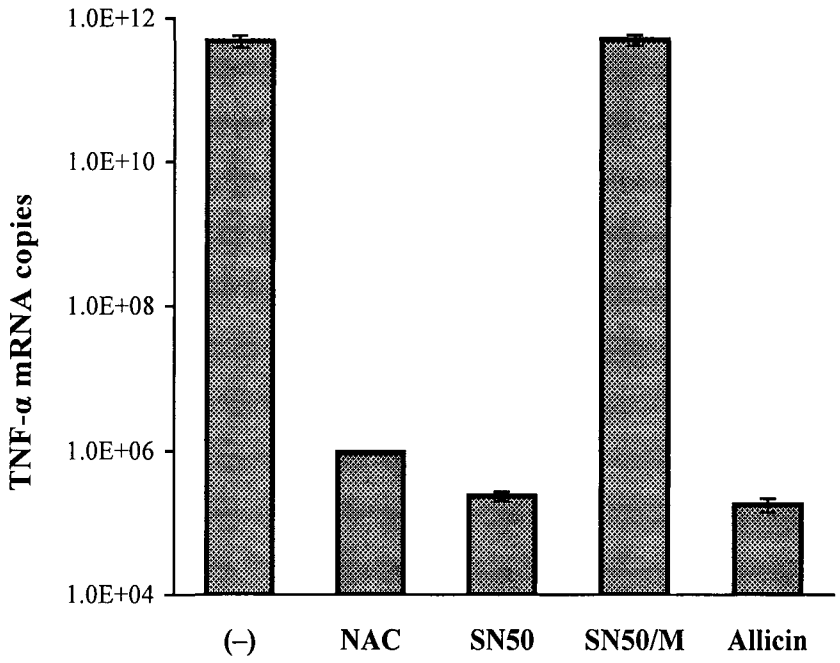


Figure 51. Real-time RT-PCR for comparative effects of NAC, SN50 and allicin on expression of TNF-α mRNA: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) in the presence or absence (–) of NAC (10 mM), SN50 (100 μg/ml), SN50/M (100 μg/ml) or allicin (500 ng/ml). Total RNA was extracted at 24 hours and was assessed for expression of TNF-α mRNA. Data represent mean ± SEM of 6 experiments.

We also probed the comparative effects of SN50 versus allicin on TNF-α and *M. tuberculosis* 85B mRNA expression in *M. tuberculosis*-infected monocytes at 24 hours of infection. SN50 (100 μg/ml) was added to monocytes 3 minutes prior to *M. tuberculosis* infection. Control cultures did not receive SN50. At 24 hours, SN50 suppressed endogenous TNF-α mRNA expression in *M. tuberculosis*-infected monocytes by around 6 logs ($P<0.001$), in comparison to control cultures devoid of SN50 pre-treatment (Fig. 51). Thus, allicin proved to be around 0.3 logs more effective than SN50. The IC_{50} value calculated for TNF-α inhibition in *M. tuberculosis*-infected monocytes by SN50 was 90 μg/ml. To ensure that cellular inhibition was not non-specific, we compared the effect of SN50 with its inactive analogue, SN50/M at the same concentration. SN50/M did not affect TNF-α mRNA expression ($P>0.05$) (Fig. 51).

Addition of H_2O_2 to *M. tuberculosis*-infected monocytes resulted in a further augmentation of ~1.2 logs in TNF-α mRNA expression (Fig. 52), compared to control infected monocytes devoid of H_2O_2 treatment. Conversely, addition of NAC, SN50 or allicin in H_2O_2-challenged *M. tuberculosis*-infected monocytes revealed a suppression by ~5 logs ($P<0.001$), 7.7 logs ($P<0.001$) and ~8 logs ($P<0.001$), respectively, in comparison to corresponding control cultures. SN50/M failed to show any effect on TNF-α mRNA expression (Fig. 52).

Thereafter, an attempt was made to evaluate the comparative modulatory effects of NAC and SN50 versus allicin on *M. tuberculosis* 85B gene expression in *M. tuberculosis*-infected monocytes at 24 hours of infection. As evident from Fig. 53, the *M. tuberculosis* 85B:16S ratio recorded was 0.088 in *M. tuberculosis*-infected monocytes at 24 hours, whereas on addition of either 10 mM NAC, 100 μg/ml SN50, or 500 ng/ml of allicin, the 85B:16S ratio was downregulated to 0.001 ($P<0.001$), 0.0003 ($P<0.001$), and 0.0002 ($P<0.001$), respectively, in *M. tuberculosis*-infected monocytes. No effect on 85B mRNA expression was found with SN50/M. Furthermore, the data depicted in Fig. 54 revealed a significant effect of NAC, SN50 and allicin in the presence of H_2O_2 on 85B:16S ratio in *M. tuberculosis*-infected monocytes. The 85B:16S ratio was found to be 0.088 in *M. tuberculosis*-infected monocytes. On addition of 10 nM H_2O_2, the 85B:16S ratio was augmented to 0.348 ($P<0.001$). On the other hand, the

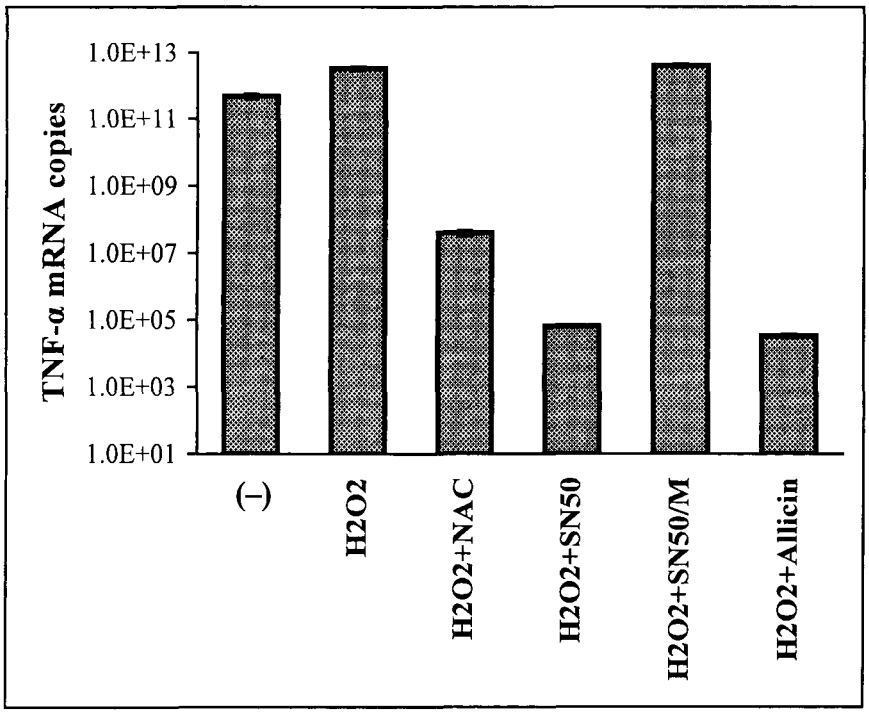


Figure 52. Real-time RT-PCR for comparative effects of NAC, SN50 and allicin in the presence of H_2O_2 on expression of TNF-α mRNA: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) in the presence or absence (–) of H_2O_2 (10 nM), NAC (10 mM) + H_2O_2 (10 nM), SN50 (100 μg/ml) + H_2O_2 (10 nM), SN50/M (100 μg/ml) + H_2O_2 (10 nM) or allicin (500 ng/ml) + H_2O_2 (10 nM). Total RNA was extracted at 24 hours and was assessed for expression of TNF-α mRNA. Data represent mean ± SEM of 6 experiments.

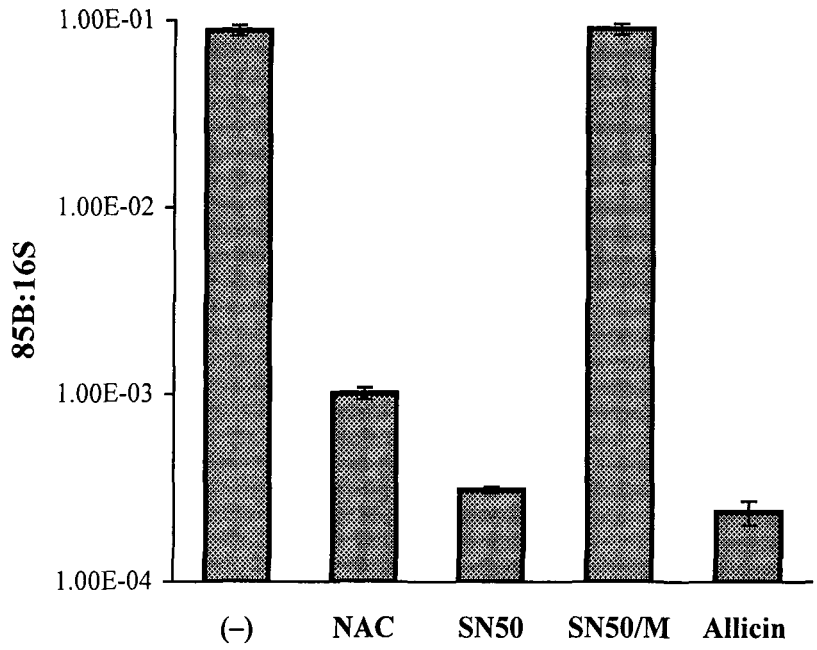


Figure 53. Real-time RT-PCR for comparative effects of NAC, SN50 and allicin on *M. tuberculosis* 85B mRNA expression: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) in the presence or absence (–) of NAC (10 mM), SN50 (100 µg/ml), SN50/M (100 µg/ml) or allicin (500 ng/ml). Total RNA was extracted at 24 hours and was assessed for expression of *M. tuberculosis* 85B:16S ratio. Data represent mean ± SEM of 6 experiments.

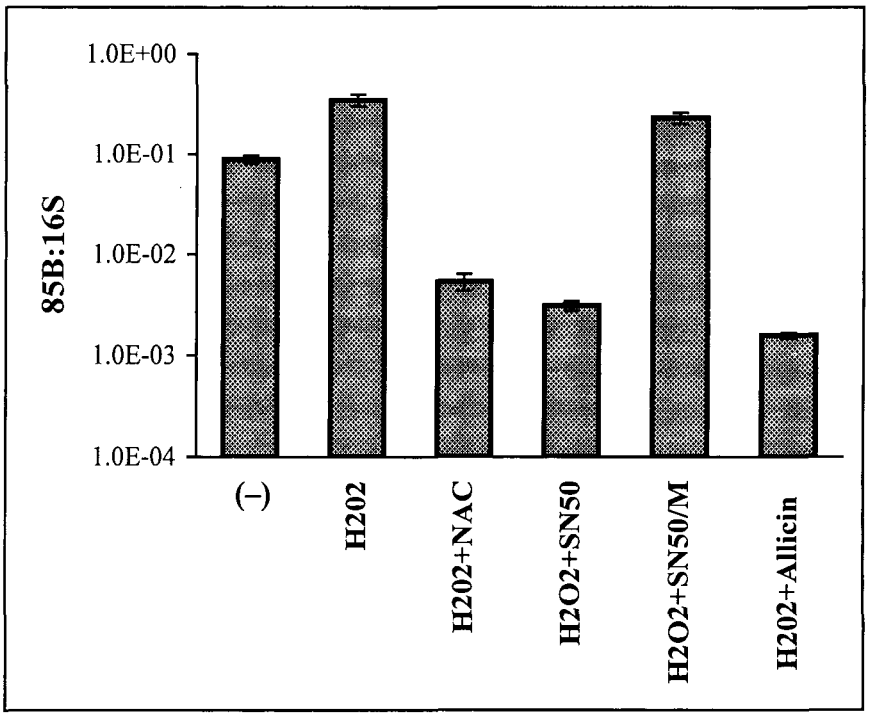


Figure 54. Real-time RT-PCR for comparative effects of NAC, SN50 and allicin in the presence of H_2O_2 on *M. tuberculosis* 85B gene expression: Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell) in the presence or absence (–) of H_2O_2 (10 nM), with NAC (10 mM), SN50 (100 μg/ml), SN50/M (100 μg/ml) or allicin (500 ng/ml). Total RNA was extracted at 24 hours and was assessed for expression of *M. tuberculosis* 85B:16S ratio. Data represent mean ± SEM of 6 experiments.

85B:16S ratio was reduced to 0.005 ($P<0.001$), 0.003 ($P<0.001$), and 0.002 ($P<0.001$) with NAC, SN50 and allicin, respectively, in *M. tuberculosis*-infected monocytes co-cultured with H_2O_2 for 24 hours (Fig. 54). The results obtained here are again suggestive of allicin to be the most potent inhibitor of host TNF-α and *M. tuberculosis* 85B gene expression in *M. tuberculosis*-infected monocytes in 24 hours cultures.

Modulation of GPx activity in *M. tuberculosis*-infected monocytes:

GPx activity was determined in *M. tuberculosis*-infected monocytes co-cultured for 24 hours with or without NAC, SN50, SN50/M and allicin. Uninfected and *M. tuberculosis*-infected, but non-treated, monocytes served as controls. GPx activity was suppressed by ~41% ($P<0.001$) in *M. tuberculosis*-infected monocytes relative to uninfected monocytes. Moreover, in comparison to *M. tuberculosis*-infected monocytes, addition of allicin (500 ng/ml), NAC (10 mM) and SN50 (100 μg/ml) enhanced GPx activity by ~43%, 40% and 37% ($P<0.001$ for all), respectively (Fig. 55). A nearly similar trend in GPx activity was also observed when *M. tuberculosis*-infected monocytes were cultured in the presence of 10 nM H_2O_2 with allicin, NAC and SN50, respectively (Fig. 55). Thus, the results obtained show that allicin proved to be a potent enhancer of GPx activity in *M. tuberculosis*-infected monocytes, both in the presence or absence of H_2O_2.

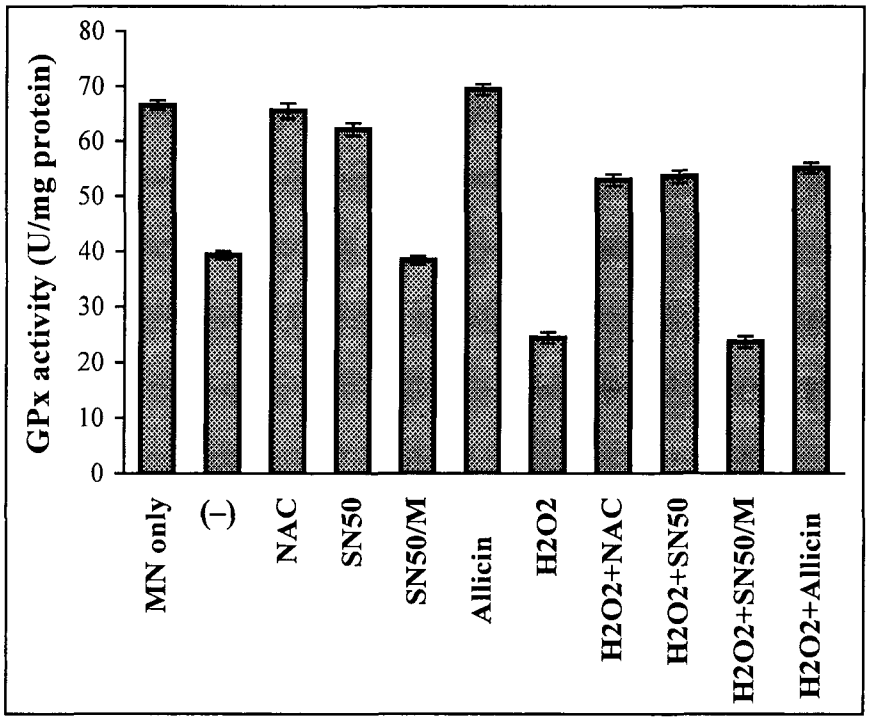


Figure 55. Modulation of glutathione peroxidase (GPx) activity. Human monocytes were infected with *M. tuberculosis* H37Rv (1:1 bacteria/cell). Some cultures were not infected (MN only) and served as controls. Cultures then received either NAC (10 mM), SN50 (100 μg/ml), SN50/M (100 μg/ml), allicin (500 ng/ml), H_2O_2 (10 nM), NAC (10 mM) + H_2O_2 (10 nM), SN50 (100 μg/ml) + H_2O_2 (10 nM), SN50/M (100 μg/ml) + H_2O_2 (10 nM) or allicin (500 ng/ml) + H_2O_2 (10 nM). GPx activity was determined in culture supernatants at 24 hours. Data represent mean ± SEM of 4 experiments.

Discussion

Tuberculosis remains a major global concern, with a staggering two million fatalities annually due to infection by the exclusively human pathogen, *Mycobacterium tuberculosis.* Declared a global health emergency more than a decade ago (WHO, 2002), tuberculosis resurgence is assuming threatening proportions. Among the important reasons for the causative killer parasite spiralling out of control at an alarming rate is attributed to the emergence of multidrug-resistant (MDR) strains and the AIDS epidemic (Toossi et al., 2004b). Despite current treatment regimens, tuberculosis continues to confound attempts at control, fuelling an urgent need for developing novel therapeutic strategies.

The initial interaction (both, phagocytic and non-phagocytic) of *M. tuberculosis* with mononuclear phagocytes gives rise to a cytokine profile that is dominated by TNF-α, a pro-inflammatory cytokine (Hirsch et al., 1994; Means et al., 2001). TNF-α is present at the site of active *M. tuberculosis* infection in humans, regardless of the stage of mycobacterial infection (Schwander et al., 2000; Hirsch et al., 2001). TNF-α is a pleiotropic cytokine, and its role in harmful or beneficial inflammatory processes is complex (Keane, 2005). Although this cytokine is involved in multiple cell regulatory and differentiation processes leading to immunity in tuberculous infection, however, TNF-α also mediates effects deleterious to the host contributing to the pathophysiology of tuberculosis. High levels of the cytokine at the site of infection induce an excessive damaging inflammatory response that overwhelms its beneficial effects (Bekker et al., 2000).

Of the various *M. tuberculosis* genes shown to be upregulated after *M. tuberculosis* infection of human mononuclear cells, the 85B gene was expressed most frequently (Graham and Clark-Curtiss, 1999). *M. tuberculosis* 85B is a predominant protein produced during human *M. tuberculosis* infection (Salata et al., 1991); however its role in tuberculosis pathogenesis is not clear and warrants further investigation. At least with regard to mycolyl transferase activity, which underlies cell-wall biosynthesis, it appears that both *M. tuberculosis* 85A and 85B are redundant (Puech et al., 2002). In sputum from patients with tuberculosis, levels of *M. tuberculosis* 85B protein and mRNA correlate with *M. tuberculosis* growth, and maintenance of 85B levels correlates with a lack of response to therapy (Wallis et al., 1998; Desjardin et al., 1999). The

expression of antigen 85B in *M. tuberculosis*-infected monocytes correlates positively with both the amount of secreted TNF-α and subsequent intracellular mycobacterial growth (Wilkinson et al., 2001). Interestingly, although many mycobacterial components induce TNF-α in mononuclear phagocytes (Barnes et al., 1992; Wallis et al., 1993; Toossi, 2000), only the members of 85 complex interact with host fibronectin (Abou-Zeid et al., 1988). Moreover, binding of 85B to fibronectin enhances the expression of TNF-α in monocytes (Aung et al., 1996). Therefore, the role of *M. tuberculosis* 85B in intracellular infection may be the maintenance of an inflammatory response. In addition, *M. tuberculosis* 85 complex may act as an intermediary to synthesis of trehalose dimycolate, which enhances the host inflammatory response (Lima et al., 2001).

It has been previously shown that infection of human alveolar macrophages with *M. tuberculosis* induces *M. tuberculosis* 85B and, TNF-α, both at mRNA and protein levels, which may be important to the immunopathogensis of disease (Islam et al., 2004). The rationale for the use of monocytes in this study is based on the fact that *in vivo*, the initial interaction of *M. tuberculosis* is with residential alveolar macrophages. If initial pulmonary defenses are overcome, successful control of the infection depends upon recruitment of blood monocytes, which are activated by cytokines, and form a granuloma restricting the growth and spread of this pathogen. Whereas the interaction of *M. tuberculosis* with more mature alveolar macrophages occurs at the very initiation and all through *M. tuberculosis* infection, the continuous recruitment of monocytes (Peters et al., 2001) to sites of *M. tuberculosis* infection ascertains that this less mature cell type interacts with *M. tuberculosis* intensely also. Thus, the cytokines and activation profile of both cell types need to be considered to develop a better understanding of host-*M. tuberculosis* interactions in situ. As mononuclear cells are recruited to sites of *M. tuberculosis* infection, their *in vitro* functional capacities may reflect in situ immune responsiveness (Toossi et al., 2004a).

Although attenuation of the biological activity of TNF-α has lately become an important therapeutic intervention in the management of a wide variety of chronic inflammatory diseases (Shanahan and St Clair, 2002), a growing body of clinical evidence indicates that neutralization of TNF-α is associated with an increased risk of

opportunistic infections, including mycobacterial diseases (Dinarello, 2003). In view of this, modulation of TNF-α release is being proposed as the basis for novel therapeutic approaches (Warwick-Davies et al., 2001). Focus has now shifted to development of compounds from natural sources that have antimycobacterial activity. By boosting host immunologic responsiveness, these compounds may be particularly useful in the treatment of drug-resistant tuberculosis. Our study involves the incorporation of such a compound, namely, allicin in garlic, as the natural herbal component for tuberculosis management.

Allicin (diallyl thiosulfinate) is the major biologically active component and thiosulfinate compound of freshly crushed garlic. It has been reported to enhance the actions of antibiotics such as chloramphenicol and streptomycin against *M. tuberculosis*. Also, it has been shown to have a broader range of bactericidal powers than penicillin: 1 mg allicin has been reported to have the equivalent antibacterial power of 15 standard units of penicillin (Koch and Lawson, 1996). The antimicrobial activity of allicin is considered to depend on its inhibitory effects on certain thiol-containing enzymes via strong SH-modifying properties, as reflected by the production of S-allylmercaptocysteine (Ankri and Mirelman, 1999; Rabinkov et al., 2000). ESR and spin trapping technique demonstrated that allicin and its precursor, alliin (+S-allyl-L-cysteine sulfoxide), possess significant antioxidant activity. In addition, it has been reported that allicin scavenges $OH^{\cdot}$ and inhibits lipid peroxidation (Prasad et al., 1995). Furthermore, it has been reported that experiments with human umbilical vein endothelial cells (HUVEC) showed that garlic extract and S-allylcysteine reduce hydrogen peroxide or tumor necrosis factor-induced NF-κB activation (Geng et al., 1997; Ide and Lau, 2001). Allicin has also been reported to exhibit its antimicrobial activity by a rapid inhibition of RNA synthesis (Feldberg et al., 1988).

In the present study, first we probed the reactivity of sera from tuberculosis patients against both, sonic extracts as well as MTCF protein antigens of cultured H37Rv and found a remarkably high magnitude of immuno-binding of both, intracellular protein antigens as well as secretory MTCF protein antigens, with tuberculosis sera. In addition, specificity of TB-IgG against *M. tuberculosis* protein

antigens was of an appreciably high degree, as evidenced by 50% inhibition being achieved at extremely low inhibitor concentrations. Further characterization of tuberculosis sera used in our study revealed elevated basal levels of TNF-α and circulating *M. tuberculosis* antigen 85 complex, which was consistent with earlier studies. Monocytes from patients with active tuberculosis were also characterized similarly. Real-time RT-PCR was employed to evaluate TNF-α and *M. tuberculosis* 85B mRNA expression, and both were found to be elevated appreciably. Furthermore, supernatants of monocyte cultures from tuberculosis patients also exhibited appreciable levels of soluble TNF-α and secreted antigen 85 complex proteins.

After establishing the virulence of the pathogenic *M. tuberculosis* strain used in our laboratory, we examined the effectiveness of allicin as a natural antimycobacterial compound, focussing on its role as an antagonist of *M. tuberculosis* 85B, in H37Rv cultures. Previous reports employing concentrations higher than those used in our study, have shown a suppression in *M. tuberculosis* growth. For this reason, we selected a lower allicin concentration that was both non-toxic and also did not affect *M. tuberculosis* growth, as evidenced by an insignificant effect on the expression of *M. tuberculosis* housekeeping gene (16S rRNA). The dose-response studies clearly demonstrated downregulation of *M. tuberculosis* 85B mRNA expression in H37Rv cultures.

Electrophoresed gel results clearly indicate the decreased expression of antigen 85 complex (30/31 kDa) in *M. tuberculosis* cultures treated with varying doses of allicin. ELISA results for secreted antigen 85 complex in *M. tuberculosis* culture supernatants also indicate the decreased secretion of antigen 85 complex when co-cultured with varying doses of allicin. To have further insight, we further designed immunoassays to study the effect of allicin on the immuno-interaction of antibodies present in sera of patients with tuberculosis against protein antigens in *M. tuberculosis* sonic extract as well as in MTCF. It was determined that allicin appreciably decreased the specificity of TB-IgG for *M. tuberculosis* protein antigens in both, sonic extracts as well as MTCF, lending support to the inhibitory effect of allicin. A concentration of 250 and 500 ng/ml of allicin proved to be a potent inhibitor of *M. tuberculosis* 85B mRNA as well as antigen 85B protein expression in *M. tuberculosis* cultures.

Next, we examined the interaction of *M. tuberculosis* with human monocytes, focussing on the relationship between expression of TNF-α and *M. tuberculosis* 85B mRNA, with or without modulators. *M. tuberculosis* induces high amounts of TNF-α (Hirsch et al., 1994; Silver et al., 1998), and the success of intracellular growth of virulent *M. tuberculosis* has been in part attributed to the capacity to induce TNF-α. In agreement with previous findings, our study also shows a time-dependent increase in TNF-α mRNA expression and protein production, reaching a maximum 24 hours post-infection. Interestingly, *M. tuberculosis* 85B mRNA expression and protein production were increased well beyond 24 hours of infection. Our results demonstrate that the induction of TNF-α and *M. tuberculosis* 85B mRNA expression mutually corresponded at t_0 and 24 hours. Thus, stronger cell activation, as has been reflected by induction of TNF-α, seems to be associated with increased expression of 85B gene. In support of this finding, exogenous TNF-α was also found to significantly induce the expression of both TNF-α and *M. tuberculosis* 85B, in parallel. These results are suggestive of a modulatory role being exerted by *M. tuberculosis*-induced TNF-α, in monocyte cultures, over the expression of *M. tuberculosis* 85B during the initial 24 hours of infection. This seems to be further reinforced by a significant down-modulation in the expression of either gene following neutralization of TNF-α by sTNFR-I and sTNFR-II.

It is to be pointed out that TNF-α may not be involved in sustaining the continued expression of *M. tuberculosis* 85B with time. The maintenance of 85B expression at time points beyond 24 hours, when TNF-α activity is reduced, suggests the involvement of alternative mechanisms as has been previously observed in macrophages (Islam et al., 2004). It has to be noted that expression of the TNF-α gene and production of its protein are under strong intracellular control mechanisms. The TNF-α mRNA expression at 24 hours showed a positive linear correlation with the secretion of antigen 85B into parallel cultures. An effect of cytokines released by *M. tuberculosis*-infected monocytes on intracellular expression of *M. tuberculosis* gene throughout infection of mononuclear phagocytes needs to be considered. The 85B:16S ratio at 24 hours showed a positive linear correlation with the secretion of TNF-α into parallel cultures.

Cellular signalling by TNF-α is mediated mainly through activation of NF-κB (Baeuerle and Baltimore, 1996). In turn, activation of NF-κB and other pathways sustain TNF-α activity (Ropert et al., 2001). To assess the role of NF-κB in the expression of TNF-α in *M. tuberculosis*-infected monocytes, we employed SN50, an inhibitor of NF-κB. From our results, it was apparent that the induction of TNF-α and *M. tuberculosis* 85B expression in *M. tuberculosis*-infected monocytes was mediated through activation of NF-κB, because both mRNAs were suppressed when SN50 was present in cultures. The inactive analogue of SN50 did not have any effect. Thus, as previously reported in macrophages (Islam et al., 2004), this study also shows that cellular activation is associated with augmentation of expression of both TNF-α and *M. tuberculosis* 85B in *M. tuberculosis*-infected monocytes

Putative and effective host defense mechanisms by innate immune cells to *M. tuberculosis* within the phagolysosomes of activated macrophages include the production of reactive oxygen (ROI) and reactive nitrogen intermediates (RNI). Phagocytosis of microbes as well as cellular activation activates ROIs (Takao et al., 1996), and H_2O_2, a product of the ROI pathway activates the expression of iNOS and production of NO (Han et al., 2001). Both ROI and RNI are downstream mediators of macrophage-activating cytokines and are thought to be microbicidal. Activation of iNOS and production of NO may be important in the final containment of *M. tuberculosis* by macrophages (MacMicking et al., 1997). However, *M. tuberculosis* has evolved resistance mechanisms against both, ROI (Hillas et al., 2000) and RNI (St John et al., 2001).

Regarding antioxidant studies, here, when the activation of RNI and ROI was inhibited by NMMA, NAC, or oATP, the expression of both TNF-α and *M. tuberculosis* 85B was significantly downregulated in monocytes infected with *M. tuberculosis*. Our results have substantiated these observations by using agents that release oxygen radicals (NADPH and H_2O_2) or NO (SNP or NOC-9). In *M. tuberculosis*-infected monocytes, these reagents increased expression of both TNF-α and *M. tuberculosis* 85B. Thus, both RNI and ROI, induced early after *M. tuberculosis* infection of monocytes, activate expression of *M. tuberculosis* gene.

Thus, our study demonstrates that *M. tuberculosis*-infection of monocytes leads to a concomitant induction of TNF-α, and expression of the *M. tuberculosis* 85B gene. Based on these findings, we probed here the regulation of TNF-α and 85B activation in *M. tuberculosis*-infected human monocytes, by allicin.

Since higher doses of allicin (>100 μM) have previously proven toxic by various investigators (Ankri et al., 1997), cell viability and potential cytotoxicity were determined for the lower concentrations employed in this study using trypan blue and MTT assays, where viability of ~98-99% was observed. Interestingly, no effect of allicin was observed on human housekeeping genes like R18 and β-actin, thereby demonstrating that the effect of allicin was not mediated by cellular death, but rather by specific inhibition of expression as well as secretion of TNF-α and *M. tuberculosis* 85B. It is to be pointed out here that allicin had no inhibitory effect on the intracellular growth of *M. tuberculosis* as revealed by the insignificant effect of allicin on 16S rRNA expression.

Allicin has earlier been reported to inhibit spontaneous and TNF-α induced secretion of pro-inflammatory cytokines and chemokines from intestinal epithelial cells (Lang et al., 2004). To the best of our understanding, we show for the first time that allicin exerts potent anti-inflammatory effects on host mononuclear cells infected with *M. tuberculosis* as evidenced by a strong inhibition of the pro-inflammatory cytokine TNF-α and in turn, of *M. tuberculosis* 85B. The results indicate an appreciable suppression in soluble TNF-α secretion as well as endogenous TNF-α mRNA expression by ~7.1 logs and 8.1 logs with 250 and 500 ng/ml of allicin, respectively in *M. tuberculosis*-infected monocytes. A parallel dose-response effect of allicin was found on *M. tuberculosis* 85B protein secretion as well as on 85B mRNA expression in *M. tuberculosis*-infected monocytes.

TNF-α production in monocytes is regulated at multiple intracellular levels, beginning with transcription (Raabe et al., 1998). Augmented expression of TNF-α mRNA (Friedland et al., 1992) and activation of a relevant transcription factor, NF-κB (Toossi et al., 1997) have been reported in monocytic cells infected with *M. tuberculosis*. Induction of TNF-α expression was mediated through activation of NF-κB, as evidenced by the suppression of TNF-α mRNA in the presence of SN50, an

inhibitor of NF-κB. On the contrary, SN50/M, an inactive analogue of SN50, failed to show any such effect. It has been well established that TNF-α induced nuclear translocation of NF-κB is inhibited by SN50 peptide as demonstrated in EMSA (Lin et al., 1995). In view of it, our data demonstrated that this effect involved inhibition of the NF-κB pathway induced by allicin, probably by inhibiting the degradation of IκBα. The NF-κB heterodimer is retained in the cytoplasm in an inactive form through association with one of the IκBs inhibitory proteins. As a consequence of stimulation by TNF-α, IκBα is phosphorylated by a specific kinase complex (IKK) leading to its ubiquitination and subsequent proteolysis by the 28S proteosome (Lang et al., 2004; Li and Verma, 2002). The degradation of IκB releases active NF-κB, which translocates to the nucleus and regulates gene expression by binding to κB binding sites or by interacting with other transcriptional factors (Brown et al., 1995). Since a number of genes involved in inflammatory responses are regulated by NF-κB pathway, thus a high magnitude downregulation of the NF-κB pathway by allicin would predictably reduce the elaboration of NF-κB-mediated TNF-α mRNA expression and thereby, 85B mRNA expression. In addition, allicin exerted a higher degree of neutralizing effects than NAC and anti-TNF-α antibodies on TNF-α-induced actions in *M. tuberculosis*-infected human monocytes.

The exact mechanism underlying the antioxidant activity of allicin still remains poorly understood. Allicin-induced enhancement of glutathione peroxidase activity has been reported (Perchellet et al., 1986; Bryk et al., 2002). In continuation to the above, a role for the antioxidant enzyme glutathione peroxidase (GPx) in infected mononuclear cells was investigated with respect to various modulators employed in this study. Glutathione directly reacts with ROS, and GPx catalyzes the removal of hydrogen peroxide (Mesiter and Anderson, 1983). Decrease in GPx activity indicates impairment of hydrogen peroxide-neutralizing mechanisms (Rukmini et al., 2004). Here, we observed a decline in GPx activity in *M. tuberculosis*-infected monocytes that were untreated or treated either with H_2O_2, SN50/M or H_2O_2+SN50/M, thereby concurring with earlier reports that substantial amounts of ROS are being generated in cells infected with *M. tuberculosis* due to cellular activation (Islam et al., 2004). Enhancement of GPx activity in *M. tuberculosis*-infected monocyte cultures after addition of NAC, a precursor of the *in vivo* antioxidant glutathione, indicates reversal

of impaired neutralizing mechanisms. Surprisingly, GPx activity was observed to be further augmented when allicin was co-cultured instead of NAC, indicating allicin to be an effective natural antioxidant combating ROS, generated as a consequence of cellular activation in *M. tuberculosis*-infected mononuclear phagocytes.

Thus, in summary, the present study shows that in human monocytes early after infection with *M. tuberculosis*, a vicious circle may exist in which expression of host inflammation and mycobacterial products amplify one another. Our study also shows enhancement of GPx activity by allicin, which correlated inversely with the downregulation of TNF-α and *M. tuberculosis* 85B mRNA expression as well as TNF-α and *M. tuberculosis* 85B protein expression in monocytes infected with *M. tuberculosis*.

In conclusion, it can be inferred from the present study that:

- There exist high basal levels of TNF-α and *M. tuberculosis* 85B proteins in sera as well as in monocyte cultures of tuberculosis patients. Furthermore, monocyte cultures of tuberculosis patients also show high basal levels of TNF-α and *M. tuberculosis* 85B mRNA.
- Allicin suppresses the expression of *M. tuberculosis* 85B mRNA and the secretion of *M. tuberculosis* 85B protein in *M. tuberculosis* (H37Rv) cultures.
- Activation of monocytes by *M. tuberculosis* induces the expression of both TNF-α and *M. tuberculosis* 85B mRNA.
- Both RNI and ROI, induced early after *M. tuberculosis* infection of monocytes, increase expression of both TNF-α and *M. tuberculosis* 85B mRNA.
- Activation of monocytes by *M. tuberculosis* initiates a cascade of events whereby a vicious circle may exist in which expression of host inflammation and mycobacterial products amplify one another.
- 500 ng/ml allicin exhibited no toxic effect on the viability of human monocytes. Moreover, this concentration of allicin has no inhibitory effect on growth of *M. tuberculosis*.
- Allicin inhibits the expression of TNF-α mRNA and its protein production in a dose-dependent manner in *M. tuberculosis* infected monocyte cultures after 24 hours of infection.
- After 24 hours of *M. tuberculosis* infection, allicin downregulates the expression of *M. tuberculosis* 85B mRNA as well as its protein secretion in monocyte cultures.
- Suppression of TNF-α and *M. tuberculosis* 85B by allicin in *M. tuberculosis*-infected monocytes is mediated mainly via NF-κB.
- Allicin enhances the glutathione peroxidase activity in *M. tuberculosis*-infected monocytes.

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